Angiotensin receptors in the cardiovascular system

Wagenaar, Lodewijk Jacob

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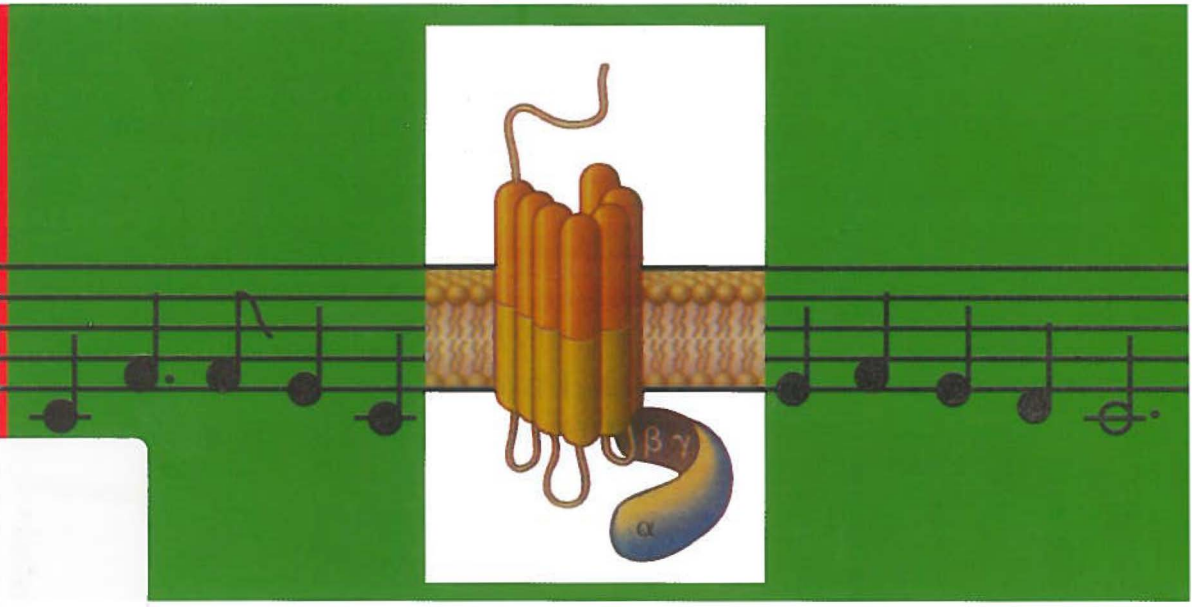

L. J. Wagenaar

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STELLINGEN

Behorende bij het proefschrift
"Angiotensin receptors in the cardiovascular system"
door Lodewijk Wagenaar

1. Angiotensine receptoren spelen een belangrijke rol bij bijna alle cardiovasculaire ziektebeelden. (*Dit proefschrift*)

2. Hoewel vaatverwijding door activatie van de AT_2 receptor als potentieel voordeel van de AT_1 receptor blokkers wordt beschouwd, is deze vaatverwijding nooit bewezen. (*Dit proefschrift*)

3. Angiotensine receptoren blokkers lijken een logische therapie ter preventie van in-stent restenose. (*Dit proefschrift*)

4. ACE-activiteit is niet voorspellend voor in-stent restenose. (*Dit proefschrift*)

5. De termen 'surmountable/insurmountable' en 'competitive/non-competitive' worden ten onrechte regelmatig door elkaar gehaald. (*Dit proefschrift*)

6. De effectiviteit van chronische therapie met angiotensine converterend enzym (ACE)-remmers komt niet doordat de conversie van angiotensine wordt geremd.

7. Het bepalen van het cholesterol gehalte bij cardiovasculaire patiënten leidt vaak tot onderbehandeling.

8. Evidence-based medicine is een zwaktebod.

9. Routinematige vasectomie is niet geïndiceerd bij operaties aan de prostaat. (*J. Wagenaar*)

10. De meest geleerde geneeskundige is niet altijd de beste arts. (*A.A. Hijmans van den Bergh, Leerboek der Inwendige Geneeskunde 1940*)

11. De overeenkomst tussen cardiologen en altviolisten is de ritmestoornis. (*J. Roelofs*)

12. Wie lang nadenkt, gebruikt niet zijn hersens maar zijn bloedsomloop. (*N. Scheepmaker*)

13. Appels en peren zijn goed vergelijkbaar.

14. De schoonheid van een altviool is aan de lage des af te meten.

RIJKSUNIVERSITEIT GRONINGEN

Angiotensin receptors in the cardiovascular system

Proefschrift

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Promotores:	Prof. dr. W.H. van Gilst Prof. dr. D.J. van Veldhuisen
Co-promotores:	Dr. A.J. van Boven Dr. J.H. Buikema

Beoordelingscommissie:	Prof. dr. P.W. Boonstra Prof. dr. P.A. de Graeff Prof. dr. H.W.M. Plokker
Paranimfen:	F.W. Asselbergs O.T.E. Jellema

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Contents

Chapter 1

Angiotensin receptors in the cardiovascular system

Lodewijk J. Wagenaar, Adriaan A. Voors,
Hendrik Buikema, Wiek H. van Gilst

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ABSTRACT

The angiotensin II receptors mediate the effects of the renin-angiotensin system, which plays an important role in cardiovascular (patho)physiology. Four types of angiotensin receptors are known, of which the AT_1 and the AT_2 receptor are the most important ones.
Stimulation of the AT_1 receptor leads to a cascade of signalling pathways in several cell types, which finally leads to processes like vasoconstriction, inflammation and proliferation. These processes are of great importance in various cardiovascular diseases, including hypertension, atherosclerosis and ventricular hypertrophy.
The AT_2 receptor is mainly expressed in the foetal stage. In adults, the AT_2 receptor is minimally expressed under normal circumstances. Its role in the adult cardiovascular system is not well established, but it appears to have opposing effects to the AT_1 receptor.
This overview discusses the pathophysiological role of the angiotensin II receptors in different cardiovascular diseases with the emphasis on the signal transduction of the AT_1 and the AT_2 receptor.

In 1836, Bright reported that patients who were dying with contracted kidneys often had a hard, full pulse and cardiac hypertrophy.[1] Sixty-two years later, Tigerstedt described the effects of a renal extract on the blood pressure. He called the substance that was responsible for this pressor response 'renin'.[2] Subsequent studies further elucidated this system that caused vasoconstriction, nowadays known as the renin-angiotensin system.[3-8] A schematic overview of the renin-angiotensin system is shown in figure 1. As angiotensin II is the most important effector peptide in this system, the receptors for angiotensin II play an important role in the effects of the renin-angiotensin system. The renin-angiotensin system has important physiologic properties in the cardiovascular system, the kidney and the brain.

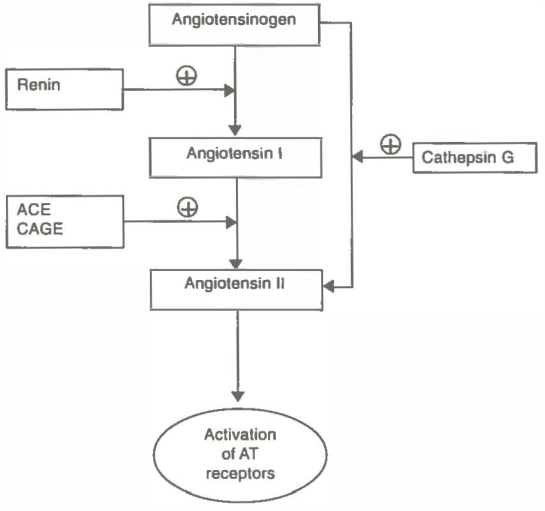

Figure 1: Schematic overview of the renin-angiotensin system
ACE: Angiotensin converting enzyme
AT: Angiotensin
CAGE: Chymostatin-sensitive angiotensin II generating enzyme

In 1989, the existence of more than one receptor for angiotensin II was demonstrated for the first time.[9] Two years thereafter, the nomenclature for the angiotensin II receptors was established as 'AT_1' for the receptor type that was inhibited by losartan and 'AT_2' for the type that was inhibited by PD123177.[10] At present, four types of angiotensin II receptors (AT_1, AT_2, AT_3 and AT_4) are known. An AT_3 receptor, also called 'non-AT_1-non-AT_2' site, was discovered in cultured neuroblastoma cells,[11] but its existence is still controversial. The AT_4 receptor has been demonstrated in human brain[11] and kidney,[12] but not in cardiovascular tissue, in contrast to bovine endothelial cells.[13] Moreover, the AT_4 receptor seems to be more specific for angiotensin IV (i.e. angiotensin 3-8) than for angiotensin II.
The aim of this review is to discuss the properties of the angiotensin II type 1 and 2 (AT_1 and AT_2) receptors in the human cardiovascular system.

THE AT_1 RECEPTOR

The human AT_1 receptor gene is located on the q22 band of chromosome 3 and was first cloned in 1992.[14,15] It contains 359 amino acids and belongs to the G protein coupled receptor superfamily that is characterised by a seven-transmembrane-domain topology. The human AT_1 receptor does not have subtypes, in contrast to rodents, in which the subtypes AT_{1a} and AT_{1b} have been described.

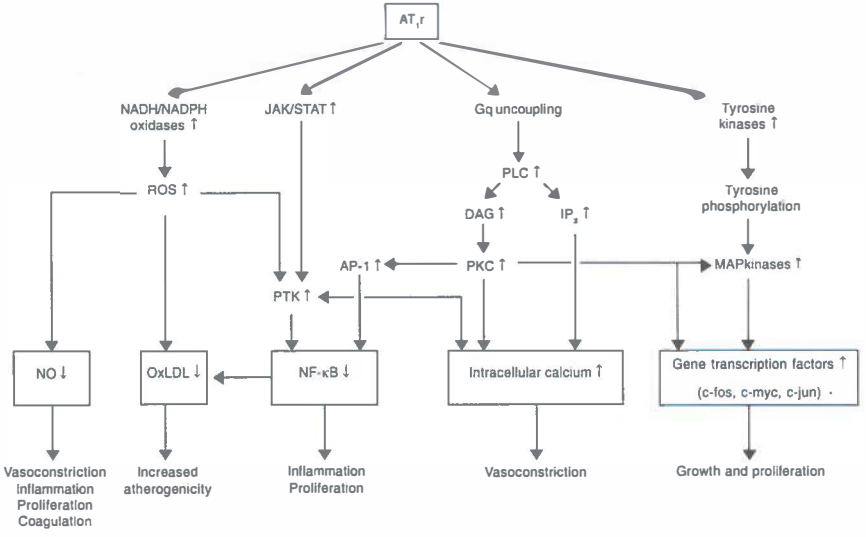

Figure 2: Schematic overview of the signal transduction of the AT_1 receptor

AP-1 : Activator protein-1
AT_1r : Angiotensin II type 1 receptor
DAG : Diacylglycerol
IP_3 : 1,4,5-inositoltriphosphate
JAK : Janus kinase
MAP : Mitogen-activated protein
NADH : Nicotinamide-adenine dinucleotide
NADPH : Nicotinamide-adenine dinucleotide phosphate
NF-κB : Nuclear factor kappa-B
NO : Nitric oxide
OxLDL : Oxidized low-density lipoprotein
ROS : Reactive oxygen species
STAT : Signal transducer and activator transcription factor
PKC : Protein kinase C
PLC : Phospholipase C
PTK : Phosphotyrosine kinase

Signal transduction

A schematic overview of the signal transduction after stimulation of the AT_1 receptor is shown in figure 2. Binding of the agonist angiotensin II to the AT_1 receptor probably leads to conformational changes in the AT_1 receptor, which leads to the uncoupling of the G_q-protein.[16] This enhances activation of phospholipase C (PLC) that on its turn generates the production of 1,4,5-inositoltriphosphate (IP_3) and diacylglycerol (DAG). Both IP_3 and DAG are able to increase the intracellular calcium concentration. IP_3 releases calcium from intracellular stores and DAG leads to influx of extracellular calcium, by stimulating protein kinase C (PKC). The increased intracellular calcium will lead to vasoconstriction in vascular smooth muscle cells.

The AT_1 receptor also regulates tyrosine kinases that appear to be required for the growth effects of angiotensin II.[17] Tyrosine kinases can cause tyrosine phosphorylation of numerous types of signalling molecules, which eventually leads to the activation of mitogen-activated protein (MAP) kinases. The MAP kinases, as well as PKC and elevated intracellular calcium, promote inducible gene transcription factors, such as c-fos, c-myc and c-jun, which leads to cell growth and proliferation.

A third pathway activated by the AT_1 receptor is the induction of the nuclear transcription factor kappa-B (NF-κB), which is mediated via Janus kinase (JAK)/signal transducer and activator transcription factor (STAT),[18] activator protein (AP-1),[19] and phosphotyrosine kinase (PTK).[20] Elevated NF-κB activity has been associated with increased macrophage infiltration, monocyte chemoattractant protein-1 (MCP-1) expression, reactive oxygen species (ROS) and with proliferation of vascular smooth muscle cells (VSMCs), and, hence, plays a role in the inflammatory component of cardiovascular diseases.[20,21]

Finally, AT_1 receptor stimulates membrane-bound NADH-/NADPH-oxidases in endothelial cells, which leads to the generation of reactive oxygen species (ROS).[22,23] ROS are capable of reducing the availability of nitric oxide (NO),[24] which leads to vasoconstriction and enhance the oxidation of LDL, which has been associated with increased atherogenicity.[25]

Receptor kinetics and regulation of the receptor

Usually the properties of the AT_1 receptor are described using a 2-state model, in which the AT_1 receptor exists in two interconvertable states.[26] One state is the so-called active state, that can be activated by angiotensin II and can mediate a biological response. In the other state, the AT_1 receptor is inactive and cannot make a biological response. Between these two states, an equilibrium exists, which can be influenced by receptor antagonists and other conditions. This theory is often used to describe interactions between agonist and antagonists. But recent studies demonstrated the existence of multiple receptor conformational states. These states are used to explain the capacity of the AT_1 receptor to couple to different signalling pathways.[27] Thomas *et al.* described a model in which the rodent AT_{1a} receptor can be in its basal state (i.e. not coupled to a ligand), in intermediate states and in different active states.[28] In the first of these active states, the AT_{1a} receptor is able to induce the induction of the PLC-mediated pathway. In another state, the receptor will be internalised, and in the last state the receptor will be phosphorylated. All these states are mutually convertible.

In several pathological states, the AT_1 receptor is upregulated in the heart and vessel walls, especially after mechanical injury, stretch or turbulence. In patients with hypercholesterolemia, the AT_1 receptor is over-expressed.[29] Also in unstable angina, the AT_1 receptor is upregulated in myocardial cells in contrast to patients with stable angina.[30] In chronic heart failure, on the other hand, the AT_1 receptor is downregulated in the heart, probably as a reaction to the increased formation of angiotensin II.[31-34] As in heart failure, atrial fibrillation leads to a downregulation of the AT_1 receptor and an upregulation of the AT_2 receptor.[35]

AT_1 receptor polymorphism

Different polymorphisms have been described concerning the AT_1 receptor, such as A39C, A153G, T573C, A1062G, A1166C, G1517T and A1878G.[36,37] Of these, the adenine/cytosine (A/C) substitution in base 1166 appears to be the most important.[38,39] The A1166C gene polymorphism has been associated with essential hypertension,[36] arterial vasoconstriction,[40] cardiac hypertrophy,[41] and aortic stiffness.[42] The increased cardiovascular risk of the A1166C polymorphism might be explained by the increased arterial response to angiotensin II.[43] It might be speculated that this polymorphism contributes to the inter-individual response to various antihypertensive drugs.[44]

THE AT_2 RECEPTOR

The angiotensin type 2 (AT_2) receptor seems to be less important than the AT_1 receptor in the (patho)physiology of the adult and has not been studied as intensively as the AT_1 receptor. The AT_2 receptor is widely expressed in foetal tissues, especially in the areas of mesenchymal differentiation. In most tissues, the AT_2 receptor strongly regresses or even disappears early after birth.[45-47] In the adult human, the AT_2 receptor has been detected in the heart (atrium and ventricle),[34,48] the adventitia of renal interlobular arteries,[49] the brain,[50] the kidney,[51] the lung,[52] the adrenal gland,[53] and the uterus.[54] In rats, the AT_2 receptor was also detected in the endothelium, but not in the VSMCs of coronary and mesenteric arteries.[55,56]

The AT_2 receptor gene was cloned one year after the AT_1 receptor, and its gene is located on the q22 band of the X-chromosome.[57-59] The AT_2 receptor contains 363 amino acids. In rat and mouse, the AT_1 receptor and the AT_2 receptor have only 24 to 33% amino acid sequence identity. The homology is mainly localised in the regions that code for the transmembrane domains. Also the amino acids that are probably responsible for the binding of angiotensin II to the AT_1 receptor are preserved in the AT_2 receptor. Like the AT_1 receptor, the AT_2 receptor belongs to the superfamily of G protein coupled receptors. At present, no subtypes of the AT_2 receptor have been described.

Signal transduction

A schematic overview of the signal transduction after stimulation of the AT_2 receptor is shown in figure 3. Binding of angiotensin II to the AT_2 receptor leads to the uncoupling of the G_I-protein.[60] The uncoupled G_I-protein activates serine/threonine phosphatase 2A (PP2A) and phosphotyrosine phosphatase (PTP), which inhibit respectively the protein-serine/threonine phosphorylation and the protein-tyrosine phosphorylation. Via these pathways, activation of the AT_2 receptor suppresses the MAPK-activation after stimulation of the AT_1 receptor, and therefore inhibits the AT_1 receptor-mediated growth and proliferation.[61,62] The AT_2 receptor is also capable of inducing NF-κB, via the intracellular production of ceramide and ROS.[20,63]

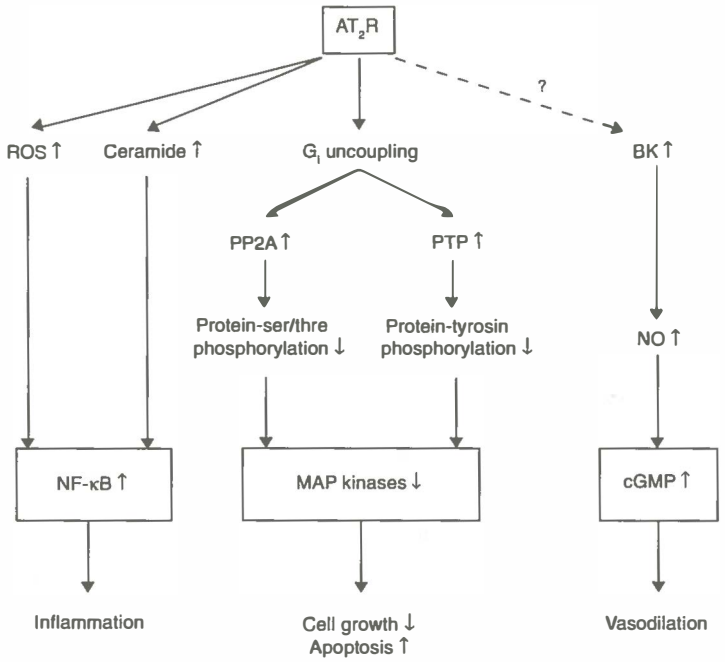

Figure 3: Schematic overview of the signal transduction of the AT_2 receptor

AT_2r	*: Angiotensin II type 2 receptor*
BK	*: Bradykinin*
cGMP	*: Cyclic GMP*
MAP	*: Mitogen-activated protein*
NF-kB	*: Nuclear factor kappa-B*
NO	*: Nitric oxide*
PP2A	*: Serin/threonin phosphatase 2A*
PTP	*: Phosphotyrosine phosphatase*
ROS	*: Reactive oxygen species*

The effects of the AT_2 receptor on the production of cGMP are still controversial. Several studies in animal models have shown that the AT_2 receptor is capable of stimulating a bradykinin/NO-cGMP pathway, which leads to vasodilation.[64-69] So far, this pathway has not been demonstrated in human vasculature.

Regulation of the receptor

In experimental settings, the AT_2 receptor can be upregulated during different (pathological) states, such as heart failure, myocardial infarction, vascular injury and sodium restriction.[68] The expression of the AT_2 receptor can be regulated by various substances like angiotensin II, adrenaline, insulin, insulin-like growth factor, fibroblast growth factor (FGF) and transforming growth factor (TGF)-β.[68,70] In a cell culture of PC12 cells, increase of intracellular calcium levels decreased the AT_2 receptor density.[71]

In human hearts, the AT_2 receptor is upregulated in several pathological states, such as ischemic heart disease, dilated cardiomyopathy and atrial fibrillation.[32,35] In heart failure the AT_2 receptor is upregulated in the fibroblasts of human ventricles, but downregulated in the cardiomyocytes.[34,72]

Physiologic effects

The AT_2 receptor mainly counteracts the effects of the AT_1 receptor. Few studies of the effects of the AT_2 receptor in humans have been performed. In different animals, the AT_2 receptor is able to inhibit proliferation of vascular smooth muscle cells (for instance after vascular injury), endothelial cells, cardiac myocytes and fibroblasts.[61,73-75] The AT_2 receptor induces a decrease in cellular matrix in the hamster heart.[73] Furthermore, the AT_2 receptor promotes vascular differentiation in VSMCs,[47] and is proapoptotic, which is an important property during the foetal development (see figure 3).[62,76] Whether the AT_2 receptor is also able to induce vasodilation in human arteries is still unknown. The vasoactive effects after activation of the AT_2 receptor differ in different animal species, and even between different branches within one species.[77-81] The potential vasodilative effect of the AT_2 receptor can therefore not be extrapolated to the human species.

The studies of Ruiz-Ortega *et al.* demonstrating that the AT_2 receptor activates NF-κB suggest a possible role of the AT_2 receptor in the pathogenesis of atherosclerosis.[20,63]

ANGIOTENSIN RECEPTORS IN CARDIOVASCULAR PATHOPHYSIOLOGY

Hypertension

The AT_1 receptor appears to be related to essential hypertension, but a causal relationship has not been established yet. The relationship is suggested, because of the efficacy of AT_1 receptor blockers in essential hypertension.[82-84] Moreover, several studies have shown that angiotensin II is able to induce different mechanisms that play a role in the complex physiology of essential hypertension.

First of all, angiotensin I I leads to direct vasoconstriction via stimulation of the AT_1 receptor in vascular smooth muscle cells. Superoxide, produced after stimulation of the AT_1 receptor, inactivates NO, and hence decreases the vasodilative capacity of the artery.[85] In endothelial cells, this stimulation also leads to the production of ROS, F_2-isoprostanes and endothelin, substances that can potentiate the vasoconstrictive effects of angiotensin II.[86] Furthermore, the activated renin-angiotensin system leads to an increased media width to lumen diameter-ratio (so-called vascular remodelling) in small arteries, independent of its effects on the blood pressure.[87] This effect is mediated via the AT_1 receptor, since it can be blocked with the AT_1 receptor blocker losartan.
Finally, the renin-angiotensin system also has an effect on blood pressure via its effects on the kidneys (water and salt control), the brain (thirst) and the sympathetic nerve system.[88]

Atherosclerosis
Angiotensin-converting enzyme (ACE) inhibitors, but recently also AT_1 receptor blockers, have proven to be effective in preventing events that are associated with atherosclerosis or vascular dysfunction.[89-91] The renin-angiotensin system plays a role in different processes in the genesis of atherosclerosis. Both angiotensin II forming enzymes (ACE, chymase) and angiotensin II itself are increased in atherosclerotic plaques.[92-94] The various effects of angiotensin II in the process of atherosclerosis are mediated via its AT_1 receptor, suggesting an important role of the ARBs in atherosclerosis.
As stated earlier, both the AT_1 and the AT_2 receptor are able to induce macrophage infiltration, via the NF-κB pathway. The infiltration of macrophages in the vessel wall is an obligatory step in the onset and progression of atherosclerosis.[95] Angiotensin II also induces the production of reactive oxygen species (ROS). ROS decrease the availability of NO, which leads to endothelial dysfunction, an early step in the atherosclerotic process.[85] ROS also stimulate macrophage-mediated oxidation of LDL.[25] Oxidized LDL may have a role in accelerated atherosclerosis, because it can promote cellular cholesterol accumulation and foam cell formation.[96,97] Interestingly, angiotensin II also increases the density of the receptor for oxidized LDL via the AT_1 receptor.[98] In humans, blockade of the AT_1 receptor reduced markers of oxidative stress in patients with essential hypertension as well as in patients with coronary artery disease.[99,100] Furthermore, AT_1 stimulation leads to proliferation and migration of vascular smooth muscle cells, and thus to neointimal formation, another important part of the atherosclerotic process.[101] PAI-1 is increased by angiotensin II, which means that angiotensin II is able to inhibit fibrinolysis, thereby predisposing to coronary thrombosis.[102] Finally, stimulation by angiotensin II leads to an upregulation of ICAM-1, VCAM-1 and E-selectine.[103-105] These molecules support monocyte adhesion to endothelial cells and may play a crucial role in the pathogenesis of atherosclerosis. The production of VCAM can be suppressed with AT_1 receptor blockade.[99,106]

Restenosis
The AT_1 receptor is involved in restenosis after vascular angioplasty in animal models. In rat carotid arteries, injury made by a balloon results in smooth muscle cell proliferation,

which is AT_1-dependent.[107] The renin-angiotensin system is also involved via its AT_1 receptor in other processes that are involved in restenosis after balloon angioplasty, as these processes are the same as the above mentioned atherosclerotic processes. But the recoil of the vessel, an important mechanism of restenosis after coronary balloon angioplasty, is not influenced by the renin-angiotensin system.

Restenosis in stented human coronary arteries differs histologically from restenosis after balloon angioplasty, as in-stent restenotic lesions contain mainly smooth muscle cells and show more active proliferation of smooth muscle cells than restenotic lesions after balloon angioplasty.[108] As stated above, stimulation of the AT_1 receptor in animal models leads to proliferation and migration of vascular smooth muscle cells, and thus to neointimal formation in the stent.[101] In specimens obtained from human coronary arteries with an atherectomy catheter, we found that smooth muscle cells of in-stent restenotic lesions contained the AT_1 receptor abundantly.[109] In humans, the AT_1 receptor blocker valsartan appears to reduce the in-stent restenosis rate.[91] As far as we know, this is the only study that investigated the effect of an AT_1 receptor blocker on in-stent restenosis in humans.

Left ventricular hypertrophy

Stimulation of the AT_1 receptor is involved in the development of left ventricular hypertrophy (LVH) in patients with hypertension as it plays a role in different processes associated with LVH, such as cardiac fibrosis, hypertrophy of cardiac myocytes and apoptosis.[110-113]

Angiotensin II leads to cardiac fibrosis by stimulating collagen synthesis and the growth of fibroblasts.[110,111] Furthermore, angiotensin II leads to downregulation of matrix metalloproteinase-1 (MMP-1) in patients with essential hypertension.[114] MMP-1 is a rate-limiting enzyme in the degradation of extracellular collagen. Indeed, the ACE inhibitor lisinopril was able to mediate regression of myocardial fibrosis in patients with mild hypertension.[115] In cardiomyopathic hamsters, an angiotensin II receptor blocker inhibited the down-regulation of MMP-1.[116]

In animal models, angiotensin II has been demonstrated to induce hypertrophy of cardiac myocytes, which was inhibited with AT_1 receptor blockers.[112,117,118] NF-κB, which is induced by angiotensin II, is critical in this hypertrophic growth.[119]

In patients with essential hypertension, the AT_1 receptor blocker valsartan produced a regression of left ventricular mass (estimated with echocardiography).[120] In another study, losartan (another AT_1 receptor blocker) reduced electrocardiographic parameters of LVH.[83] More studies have suggested the ability of different AT_1 receptor blockers to regress left ventricular hypertrophy.[121] Finally, in rat studies, angiotensin II induces apoptosis via the AT_1 receptor.[113] This has not been demonstrated in human hearts. Angiotensin II did not influence cardiac contractility in preparations of human ventricular myocytes, in contrast to some animal studies.[122,123]

Chronic heart failure

The renin-angiotensin system plays a role in chronic heart failure (CHF), since the above-mentioned processes that lead to LVH are also involved in the deterioration to the clinical

diagnosis of CHF. Moreover, peripheral vasoconstriction, another important feature of CHF, is also caused by stimulation of the AT_1 receptor.
Recently, the Val-HeFT study demonstrated a possible beneficial role for blockade of the AT_1 receptor in chronic heart failure.[124] Also in a meta-analysis it was concluded that the angiotensin receptor blockers are promising in the treatment of heart failure.[125]

Atrial fibrillation
During atrial fibrillation (AF), the angiotensin-converting enzyme (ACE) is upregulated, which could lead to an AT_1 receptor-dependent atrial fibrosis.[126] In a dog model, stimulation of the AT_1 receptor also led to the shortening of the atrial effective refractory period, which increases the inducibility and stability of AF.[127] These findings suggest the involvement of the renin-angiotensin system via its AT_1 receptor in atrial fibrillation. This is confirmed in a recent study in which the AT_1 receptor blocker irbesartan reduces recurrences of atrial fibrillation.[128]

CONCLUSION

The AT_1 receptor plays an important role in cardiovascular pathophysiology. Stimulation of the AT_1 receptor leads to a cascade of signalling enzymes, which in the end results in a decrease of nitric oxide and an increase of oxidized LDL, NF-κB, gene transcription factors and intracellular calcium. Depending on local conditions, this will lead to vasoconstriction, increased atherogenicity, inflammation, growth, proliferation and/or coagulation. These processes are crucial in the different stages of atherosclerosis and are also important in hypertension, left ventricular hypertrophy, chronic heart failure, (in-stent) restenosis and atrial fibrillation.
The AT_2 receptor has been investigated less thoroughly than the AT_1 receptor. Stimulation of this receptor leads to a decrease of MAP kinases, and probably to a increase of cGMP, resulting in a diminished cell growth, apoptosis and probably vasodilation. Hence, the AT_2 receptor appears to oppose processes that are induced by the AT_1 receptor.
Given these properties, the angiotensin II type 1 receptor blockers (ARBs) could be an effective treatment for the above-mentioned diseases. ARBs do not only work by reducing the deleterious effects of the AT_1 receptor, but probably also increase the angiotensin II concentration by a negative feedback loop. In the presence of an ARB, this increased angiotensin II will act as an AT_2 receptor agonist, thus opposing the AT_1 effects. This is a theoretical advantage compared to ACE-inhibitors.

The ARBs have already proven to be effective in hypertension. Also in heart failure and in-stent restenosis, studies are promising, but not yet conclusive. To assess the value of ARBs in heart failure, but also in secondary prevention in patients with ischemic heart diseases, more randomised trials will have to be performed. Moreover, at present there is insufficient

knowledge of the effects of stimulation of the AT_2 receptor. As long as we do not fully understand its role, we will not be able to compare the working mechanism of ACE-inhibitors and ARBs, and we will not be able to predict the effects of combination therapy with ARBs on top of an ACE-inhibitor.

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Chapter 2

Aims of this thesis

Angiotensin II receptors mediate the effects of the renin-angiotensin system, which plays an important role in cardiovascular (patho)physiology. In the previous chapter, the importance of the angiotensin II receptors in the human cardiovascular system has been described. Especially the angiotensin II type 1 (AT_1) receptor plays a pivotal role in important pathophysiological processes and diseases, such as atherosclerosis and chronic heart failure. As might be expected for such a complex enzyme system, not all properties have been elucidated yet.

The renin-angiotensin system can be influenced pharmacologically by angiotensin converting enzyme (ACE)-inhibitors and by AT_1 receptor blockers (ARBs). Both ACE-inhibitors and ARBs have proven their usefulness in various cardiovascular diseases. As ARBs have been developed later, they are not as thoroughly investigated as the ACE-inhibitors.

This thesis has two main goals:
1. to describe the (possible) role of the renin-angiotensin system in the pathophysiological process of in-stent restenosis;
2. to further expand our knowledge of ARBs in the cardiovascular system.

Histological patterns and the expression of ACE and the AT_1 receptor in both *de novo* stenotic and in-stent restenotic lesions in human coronary arteries are described in *chapter 3*. *Chapter 4* discusses the possible role of ACE in the process of in-stent restenosis.
A pharmacological comparison of different ARBs is presented in *Chapter 5*. Finally, in *chapter 6*, a study describing the effects of both an ACE-inhibitor and an ARB on endothelial function in a rat model of chronic heart failure is presented.

Chapter 3

Differential localisation of the renin-angiotensin system in *de-novo* lesions and in-stent restenotic lesions in in-vivo human coronary arteries

Lodewijk J. Wagenaar, Ad J. van Boven, Allard C. van der Wal, Giovanni Amoroso, René A. Tio, Chris M. van der Loos, Anton E. Becker, Wiek H. van Gilst

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ABSTRACT

Objective. Different components of the renin-angiotensin system (RAS) have been demonstrated in atherosclerotic plaques. However, the involvement of the RAS in in-stent restenosis is not clear. We studied the differential immunolocalisation of angiotensin converting enzyme (ACE) and the angiotensin II type 1 (AT_1) receptor in *de-novo* stenotic lesions and in-stent restenotic lesions in human coronary arteries.

Methods. Using a pullback atherectomy catheter, biopsies from *de-novo* coronary lesions (n=19) and in-stent restenotic lesions (n=19) were obtained. The biopsies were immunostained for vascular smooth muscle cells (VSMCs), macrophages, ACE and the AT_1 receptor.

Results. In biopsies from *de-novo* stenotic lesions ACE-positive macrophages were more numerous than in in-stent restenotic lesions (p=0.002). Moreover, in the latter lesions, ACE-positive macrophages decreased when the time interval of stent implantation was longer. On the other hand, in-stent restenotic lesions contained predominantly young VSMCs, which abundantly expressed AT_1 receptors.

Conclusions. Lesional ACE expression is not a prominent feature of in-stent restenotic lesions. In contrast, AT_1 receptors are abundantly expressed on young VSMCs. In *de-novo* lesions ACE and AT_1 receptors were found on macrophages and VSMCs, which were present in all specimens.

The involvement of the renin-angiotensin system (RAS) in the occurrence of atherosclerotic plaque instabilisation has already been addressed. An increased accumulation of angiotensin-converting enzyme (ACE), of angiotensin II, and of angiotensin II type 1 (AT_1) receptors has been demonstrated in human coronary arteries in the course of acute coronary syndromes, in relationship with the amount of activated macrophages present in the atherosclerotic plaque.[1-3] RAS activity in stable atherosclerotic lesions is still under investigation.[4-6]

Despite the diffuse utilisation of stents, restenosis after percutaneous coronary interventions (PCI) still develops in 10-21% after stent placement.[7] Histopathological differences suggest a different pathogenesis for in-stent restenotic and atherosclerotic *de-novo* lesions.

In *de-novo* coronary atherosclerosis, the angiotensin-converting enzyme (ACE) has emerged as one of the factors involved in the process of lesion formation, since an increased accumulation of ACE in humans has been demonstrated in atherosclerotic coronary and carotid arteries.[4-6]

In-stent restenosis seems to be caused exclusively by neointimal proliferation.[8] In-stent restenotic lesions contain mainly smooth muscle cells and show more active proliferation of smooth muscle cells than native lesions and even restenotic lesions after balloon angioplasty.[9] Given the fact that smooth muscle cell proliferation can be induced by angiotensin II via the AT_1 receptor under experimental conditions,[10-12] we investigated the potential involvement of the renin-angiotensin system in the development of human in-stent restenosis. For this purpose we used atherectomy specimens retrieved from patients with in-stent restenotic lesions, in which the immunoreactivity of smooth muscle cells and inflammatory cells with anti-ACE and anti-AT_1 receptor antibodies were evaluated. Moreover, the results were compared with atherectomies from *de-novo* atherosclerotic lesions subjected to similar immunohistochemical procedures.

METHODS

Study population

Consecutive patients with recurrent stable angina or inducible ischemia after coronary stenting, and the angiographic finding of in-stent restenosis were enrolled (n=19). In-stent restenosis was defined as an in-stent lumen diameter of less than 50% of the vessel. A second population of symptomatic patients with significant *de-novo* coronary lesions, matched for age, gender and lesion location was also selected (n=19). These patients had stable angina and type B2 or C lesions in the proximal left anterior descending artery or the ramus circumflex.

Exclusion criteria for both groups were unstable angina and/or non-Q wave myocardial infarction, total coronary occlusion, vessel reference diameter <3.0 mm, excessive tortuosity of the proximal vessel and the use of an ACE-inhibitor or an angiotensin II type 1 receptor blocker.

Table 1. Patient characteristics

	Stenosis (n=19)	Restenosis (n=19)	P
Age (years)	58	62	0.16
Cholesterol (mmol/L)	5.1	4.4	0.02
Medication (n (%))			
Aspirin	19 (100)	19 (100)	1.00
Statin	9 (47)	15 (79)	0.09
Beta blocker	16 (84)	12 (63)	0.27
Calcium antagonist	10 (52)	11 (58)	1.00
Nitrate	8 (42)	11 (58)	0.52
Risk Factors (n (%))			
Family history	7 (37)	7 (37)	1.00
Smoking	8 (42)	5 (26)	0.50
Diabetes mellitus	0 (0)	2 (11)	0.49
Hypertension	11 (58)	7 (36)	0.33
Hyperlipidemia	10 (53)	17 (89)	0.03
Time after stent placement (days (min-max))	-	201 (69-435)	-

Patient characteristics are shown in table 1. The study conforms with the principles outlined in the Declaration of Helsinki.

Tissue collection

Percutaneous coronary atherectomy was performed, both in *de-novo* lesions and in in-stent restenosis, by means of a pullback atherectomy catheter (Arrow int., Reading, PA, USA). Briefly, this 10-french compatible over-the-wire catheter is positioned beyond the lesion to treat. After the cutting blade is exposed and on-axis high-speed rotation is started, the catheter is gently withdrawn by manual pullback. This procedure allows complete circumferential cuts along the whole length of the lesion, thus providing in vivo plaque samples, suitable for histopathological analysis. In all patients, one biopsy was taken out of the culprit lesion. Coronary angiography before and after the atherectomy confirmed that the sample was taken from the lesion. After collection, the samples were immediately frozen in liquid nitrogen and stored in a –80 °C freezer.

Tissue preparation and staining techniques

Frozen specimens were oriented along their longest axis in Tissue Tek. Of each specimen, 5 µm sections serially cut were mounted in duplo on glass slides. One slide was stained with Haematoxylin & Eosin staining and consecutive serial sections were used for immuno-

histochemistry. The primary antibodies we used were reactive with epitopes specific for macrophages (CD68 (clone EBM11), DAKO, Glostrup, Denmark), α-smooth muscle cells (SMA-1 (clone 1A4), DAKO, Glostrup, Denmark), ACE (mouse antibody (clone 9B9), Chemicon, Temecula, CA, USA) and the AT_1 receptor (rabbit antibody, Biotrend, Köln, Germany). Acetone fixed sections were subjected to a three-step streptavidin-biotin alkaline phosphatase (AP) staining procedure. ACE and AT_1 receptor antibodies were incubated overnight at 4°C. Tris-buffered saline was used for washing between the subsequent incubation steps. Biotinylated goat anti-mouse immunoglobulin (1:200) or biotinylated goat anti-rabbit immunoglobulin (1:400)(both DAKO, Glostrup, Denmark) were used as second step (30 minutes room temperature), AP-conjugated streptavidin (DAKO) as third step (1:100; 30 minutes room temperature). AP enzymatic activity was visualised using the Fast Red tablet substrate system (DAKO).
Immuno doublestaining for the simultaneous identification of smooth muscle cells and macrophages was based on two primary antibodies of different IgG subclasses as described earlier.[13] Using AP as enzymatic marker macrophages were visualised in blue with Fast Blue BB/Naphthol-AS-MX-phosphate as chromogens (Sigma, ST Louis, MO, USA). Smooth muscle cells were developed in red with peroxidase as marker enzyme and 3-amino-9-ethylcarbazole (Sigma) as chromogen.
Sections of alveolar lung tissue and infarcted myocardium served as positive controls for anti-ACE and anti-AT_1 staining.

Analysis of immunostaining
A semi-quantitative score of ACE, AT_1 receptor, CD68 and SMA-1 staining was applied by two independent persons (AvdW, LJW) in order to evaluate the cell type and number of cells positive in each biopsy. These persons were blinded to the patient characteristics. The weighted kappa for interobserver variability was 0.65. The semi-quantitative score we used is as follows: 0: absent; 1: few scattered cells or clusters <5 cells; 2: <10% of cells positive; 3: 10-50% of cells positive; 4: >50% of cells positive.
An immunodouble stain for smooth muscle cells and macrophages was used to facilitate the semi-quantitative scoring of macrophages and smooth muscle cells in each biopsy, and to establish the immunolocalisation of ACE and the AT_1 receptor in different cell types. Anti-ACE and anti-AT_1 stains were not useful for doublestaining procedures in combination with cell-specific stains (CD68 and SMA-1) because of huge differences in staining intensity.

Statistical analysis
Two-tailed T-test and Chi-square tests were performed to compare continuous and categorical variables, respectively. A Spearman correlation (SPSS for Windows 10.1) was used to compare the influence of timing of the biopsy on the histological parameters. A weighted kappa coefficient (SPSS for Windows 10.1) was calculated to determine the interobserver agreement. Differences were considered significant at a level of $P<0.05$.

RESULTS

Patient groups were generally well matched with respect to their baseline characteristics, although hyperlipidemia was more frequent in patients with in-stent restenosis. Neither the presence of hyperlipidemia, nor the cholesterol level did relate to the immunohistochemical outcome parameters of this study (data not shown).

In biopsies from *de-novo* stenoses a combination of different tissue components was found, including sclerotic tissue and lipid cores. Thrombus was found in only one biopsy. In 3 specimens (16%), neointimal formation was found. All but one biopsies contained both macrophages (CD68-positive) and smooth muscle cells (SMA-1-positive), but in highly variable amounts. One biopsy contained only smooth muscle cells without CD-68-positive cells. Only three biopsies contained vascular smooth muscle cells (VSMCs), consistent with neointimal proliferation, i.e. stellate shaped smooth muscle cells embedded in abundant myxoid extracellular matrix. Anti-ACE staining was observed in macrophages only. Smooth muscle cells were always ACE-negative. Anti-AT_1 receptor staining was seen on both macrophages and smooth muscle cells, but macrophages showed higher staining intensity. Typical examples of biopsies from *de-novo* stenoses are shown in figure 1a-c.

In-stent restenotic lesions contained predominantly stellate shaped VSMCs in abundant myxoid matrix, characteristic for neointimal tissue. Macrophages were either absent (11% of the tissue specimens) or present in small clusters (89%). Also in in-stent restenosis, anti-ACE staining was only seen in the macrophages, if present, whereas macrophages and all smooth muscle cells, including the stellate VSMCs in the neointimal tissue, were AT_1 receptor-positive. Typical examples of biopsies from in-stent restenoses are shown in figure 2a-c.

Semi-quantitative analysis of immunostaining showed that in-stent restenotic lesions contained more VSMCs ($p=0.003$), but less macrophages ($p=0.002$), than *de-novo* lesions (Figure 3). Moreover, in-stent restenotic lesions showed less ACE-positive cells ($p=0.02$), but slightly more AT_1 receptor staining, allthough not significant ($p=0.16$), when compared with *de-novo* lesions (Figure 3). Therefore, the overall expression of AT_1 receptors in restenotic lesions appeared much more abundant than in *de-novo* lesions with similar clinical presentation.

The time between the stent placement and the biopsy in patients with in-stent restenosis appeared to be negatively correlated with the amount of macrophages in the in-stent restenotic plaque ($r=-0.558$; $p=0.013$), although the number of cases in this instance is very small (Figure 4). As ACE was only seen in macrophages, the timing of the biopsy is also negatively correlated to the ACE-content in the plaque ($r=-0.571$; $p=0.011$).

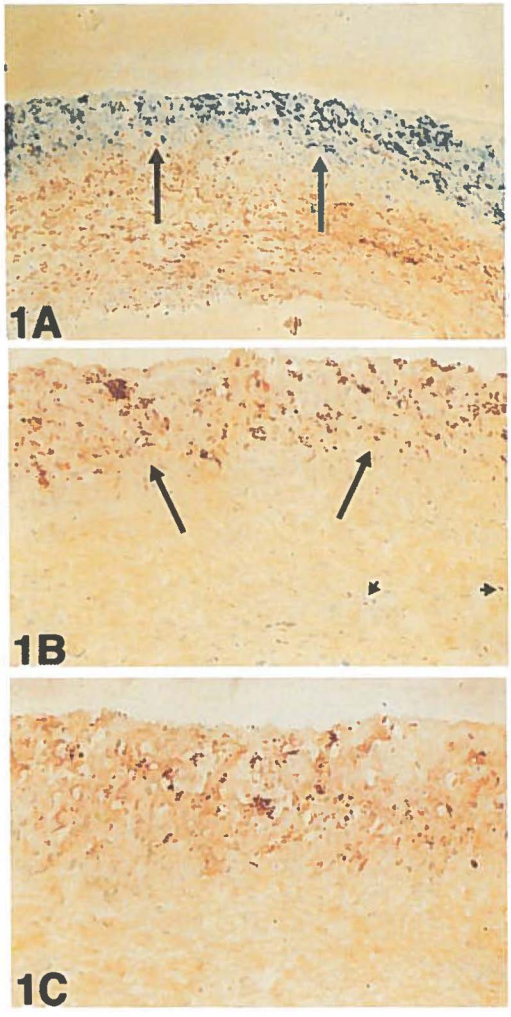

Figure 1: Immunostained sections of part of a fibrocellular area in an atherectomy specimen derived from a de-novo atherosclerotic lesion.

a. *Immunodouble stain with anti-CD68 (blue) and anti-SMA-1 (red brown) antibodies showing a band-like infiltrate of anti-CD68 positve macrophages (blue) in the upper part of the tissue (area above two arrows) and large amounts of vascular anti-SMA-1 positive smooth muscle cells (red brown) in the lower part part of the tissue.*

b. *Serial section of the same area in 1a showing angiotensin II type 1 (AT_1) receptor immunoreactivity in red of the same band of macrophages as shown in fig 1a (area above arrows) and faint AT_1 receptor immunoreactivity of smooth muscle cells (arrowheads, small red granules) with anti-AT_1 receptor antibody.*

c. *Detail of a serial section of the same macrophage rich tissue area which is indicated by arrows in 1a and b, in this figure immunostained with anti-angiotensin converting enzyme (ACE) antibody. Many macrophages show abundant cytoplasmic staining in red.*

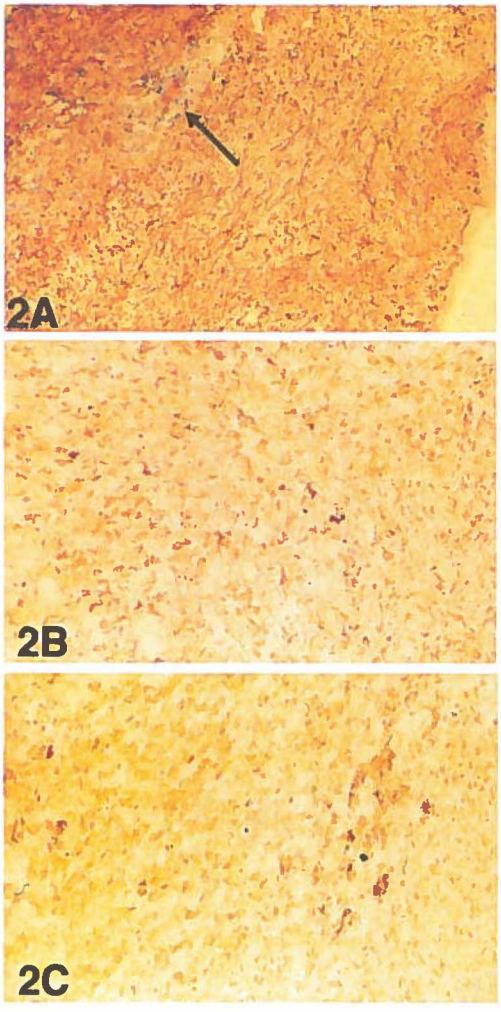

Figure 2: *Immunostained sections of part of an atherectomy specimen derived from an in-stent restenotic lesion. Photos are taken from an area containing neointimal tissue.*

a. *Immunodouble stain with anti-CD68 antibody (blue, macrophages) and anti-SMA-1 antibody (red, smooth muscle cells). The tissue composition is dominated by large amounts of spindle shaped smooth muscle cells (red). In between is a cluster of macrophages (blue stained cells) indicated by arrow.*
b. *High magnification of the smooth muscle cell (VSMC) rich neointimal tissue showing a granular type of immunostaining in red of VSMCs with anti-angiotensin II type 1 receptor antibody. Cytoplasmic staining is present in cells throughout the tissue.*
c. *High magnification of neointimal tissue, taken from a serial section stained with anti-angiotensin converting enzyme (ACE) antibody. Scarce cytoplasmic staining macrophages (red cells) are present. The majority of cells (in adjacent sections identified as VSMC, see fig 2a), of which nuclei are faintly counterstained with haematoxylin, are anti-ACE negative.*

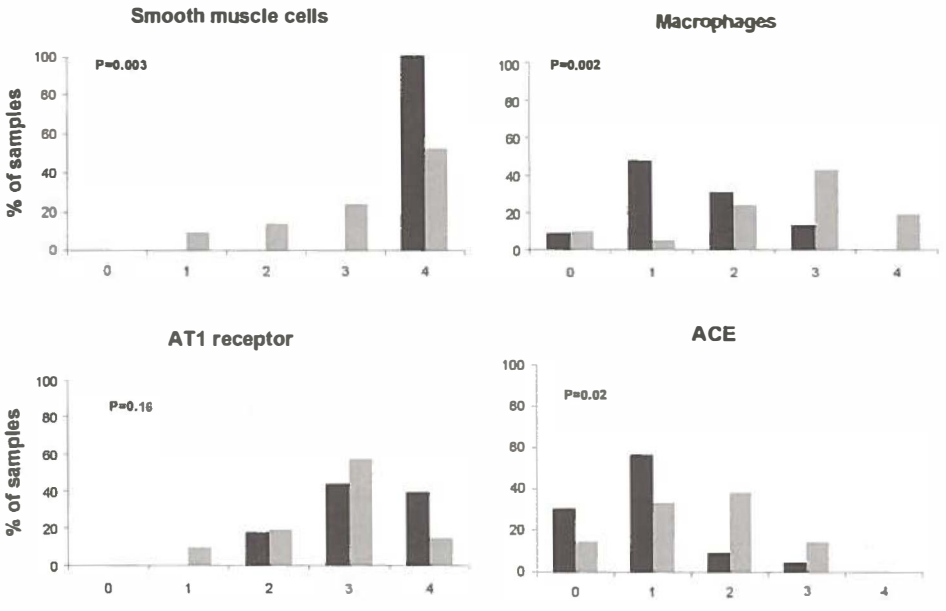

Figure 3: Semi-quantitative scores for smooth muscle cells, macrophages, angiotensin II type 1 (AT_1) receptor and angiotensin converting enzyme (ACE) in in-stent restenosis (black bars) and de-novo stenosis (grey bars).

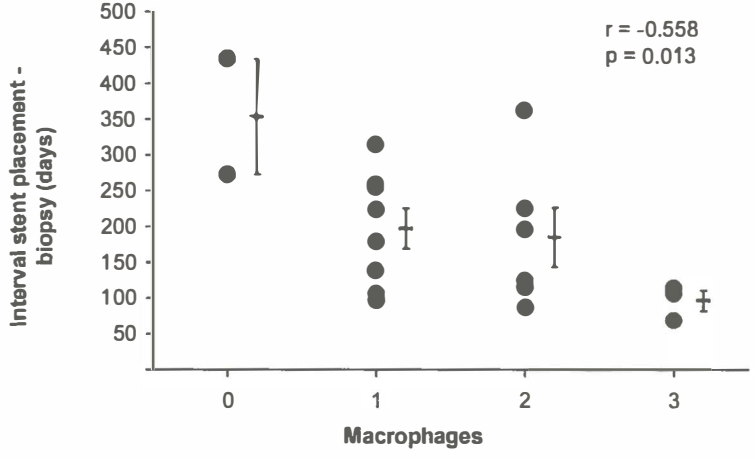

Figure 4: Inverse correlation between the amount of macrophages (semi-quantitative score) in the in-stent restenotic lesions and the time-interval between the placement of the stent and the biopsy (n=19) (mean ± SEM).

DISCUSSION

This study demonstrates the potential involvement of the renin-angiotensin system in the development of in-stent restenosis in human coronary arteries. Moreover, different patterns evolve comparing in-stent restenosis with *de-novo* stable coronary lesions. In *de-novo* stenosis, ACE was present in macrophage-rich regions in all specimens. The AT_1 receptor was found on vascular smooth muscle cells (VSMCs) and macrophages. In in-stent restenosis, ACE-staining was limited to the macrophages, if present, and hence in much lower quantities than in *de-novo* lesions. The AT_1 receptor on the other hand was more abundantly present in in-stent restenotic lesions, especially due to the large number of VSMCs.

Atherosclerosis and the renin-angiotensin system

Recent observations suggested that RAS might be involved in the occurrence of acute coronary syndromes. In fact, co-localisation of elevated ACE and angiotensin II, the RAS effector molecule in the vessel wall, in macrophages-rich areas of atherosclerotic plaques has already been demonstrated.[1,2] It is well-known that activated macrophages play a major role in plaque inflammation and subsequent disruption.[3] Moreover, several studies have shown the effectiveness of ACE-inhibitors in reducing the incidence of cardiovascular adverse events.[14,15] Although Powell *et al.* showed reduction of restenosis by ACE inhibitors in a rat model of balloon angioplasty,[16] chronic ACE inhibition did not prevent restenosis after non-stent PTCA in humans.[17,18]

In our study, ACE immunoreactivity appeared mainly as distinct granules in the cytoplasm of inflammatory cells and was present in all biopsies of *de-novo* lesions.

The pathophysiological effects of angiotensin II, the effector molecule of the renin-angiotensin system, in the vessel wall are mediated through the AT_1 receptor.[10,12,19-21] Blockade of the AT_1 receptor, as well as ACE-inhibition, could be helpful in premature atherosclerosis, as already proven in animal models and humans.[22,23] We found the AT_1 receptor to be present on both macrophages and VSMCs in the atherosclerotic plaque.

In-stent restenosis and the renin-angiotensin system

The process of in-stent restenosis has been described in several post-mortem studies.[9,24-26] The first step in the process is platelet deposition and aggegration, which is followed by a period of thrombus formation and inflammation, mainly at the sites of the struts. Smooth muscle cells are detected from 9-12 days after stent placement. After circa 30 days, neither thrombi nor acute inflammatory cells are found. At that time, chronic inflammation is still detectable around the struts, but the newly formed tissue responsible for restenosis development mainly consists of smooth muscle cells (96% of total plaque area) embedded in a proteoglycan rich matrix. Such a tissue composition is characteristic for neointima formation.

Activation of the renin-angiotensin system is one of the possible mechanisms for VSMC-proliferation. Angiotensin II is able to induce proliferation of VSMCs, a key mechanism in

in-stent restenosis, via stimulation of AT_1 receptors in experimental models.[10-12,27,28] However, apart from a case report,[29] which showed ACE-activity in restenotic material from a popliteal stent, RAS contribution to human in-stent restenosis has not been elucidated yet.
In our biopsies, the immunoreactivity with anti-ACE was restricted to inflammatory cells, and present in 74% of in-stent restenotic lesions. A higher amount of macrophages (and hence of ACE-staining) was found in in-stent restenotic lesions that had the shortest interval between the stent placement and the biopsy. This indicates that also in in-stent restenosis infiltration of inflammatory cells may play a role in the RAS-pathway. The fact that we still found macrophage-rich lesions more than 30 days after stent placement is in contradiction with the above-mentioned studies about the genesis of in-stent restenosis.
The presence of the AT_1 receptor on the abundantly present VSMCs in this tissue might indicate that the RAS plays a role in the growth and migration of VSMCs in in-stent restenosis. However, the role of ACE in the genesis of in-stent restenosis is still unclear. Several studies reported that the ACE-inhibitor quinapril reduces in-stent intimal hyperplasia, late luminal loss and restenosis rate.[30-32] Also, a recent retrospective study in 1598 patients suggested that ACE-inhibitors decrease late revascularisation after stent placement.[33] In the PARIS study, on the other hand, quinapril was not able to prevent in-stent restenosis in patients with the DD genotype of ACE.[34] It remains controversial whether patients with the DD-allele of the ACE-polymorphism are especially at risk for in-stent restenosis, due to their higher ACE-activity. The D-allele has been identified as a prognostic factor for in-stent restenosis in some studies,[35,36] but other larger studies did not confirm this.[37-39] Since the effects of the RAS are mediated via the AT_1 receptor, blockade of the AT_1 receptor could be expected to be effective in preventing in-stent restenosis. The results of the present study may endorse the outcome of a recently published study in 250 patients, which demonstrates the effectiveness of the AT_1 receptor blocker valsartan in preventing in-stent restenosis after angioplasty with stent placement in type B2/C lesions.[40] Larger studies will be necessary to confirm this hypothesis.

CONCLUSIONS

Our results are consistent with involvement of the renin-angiotenin system in stable coronary atherosclerosis. In *de-novo* lesions ACE and AT_1 receptors were found on macrophages, which were present in all specimens. All VSMCs were AT_1-positive, but less intense than the macrophages. In contrast, in-stent restenotic lesions consisted mainly of AT_1 receptor-positive VSMCs, highly characteristic for neointimal proliferation. Macrophages were present in most biopsies, but only in small amounts. Anti-ACE and anti-AT_1 receptor staining was seen on most of these macrophages. This might demonstrate the important role of the renin-angiotensin system, and especially the AT_1 receptor in in-stent restenosis.

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Chapter 4

Pre-procedural ACE-activity does not predict in-stent restenosis

Lodewijk J. Wagenaar, Braim M. Rahel, Ad J. van Boven,
Adriaan A. Voors, Allard C. van der Wal,
H.W. Thijs Plokker and Wiek H. van Gilst

ABSTRACT

Objective. Several studies indicate that ACE-activity is related to atherosclerosis. We investigated the correlation between ACE-activity, in plasma as well as in the atherosclerotic plaque, and in-stent restenosis.

Methods and results. ACE-activity was measured in blood samples from 178 patients who underwent a percutaneous coronary intervention with stent placement. During 8 months follow-up, 51 of these patients had an adverse clinical event. ACE-activity did not differ between patients with or without adverse events (21.5 vs. 23.1 nM/ml/min; P=0.36) Tissue samples were obtained with an atherectomy catheter before elective stent placement in another group of 13 patients with de novo stenosis. In this tissue, we determined the ACE-content immunohistologically. These patients were scheduled for follow-up quantitative coronary angiography after 12 months. In this group, the quantity of ACE was not correlated to the late luminal loss (0.31 vs. 0.38 mm; P=0.76).

Conclusion. In this study, pre-procedural ACE-activity, in plasma as well as in the atherosclerotic plaque, does not predict the occurrence of in-stent restenosis.

The introduction of the stent reduced the incidence of restenosis after percutaneous coronary interventions (PCI) remarkably.[1,2] But still 10-21% of the patients develops in-stent restenosis.[3] Several prognostic factors have been determined, such as length of the stent,[4] strut thickness,[5] the type of the stent,[6] diabetes,[7] and the post-procedural minimal lumen diameter.[8] Also the plaque burden outside the stent appears to correlate with the amount of neointimal proliferation inside the stent in rabbits,[9] as well as in humans.[10] In-stent restenosis is mainly caused by growth of vascular smooth muscle cells within the stent,[11] which can be induced by angiotensin II via its type 1 (AT_1) receptor.[12] In a previous study, we demonstrated that vascular smooth muscle cells in human in-stent restenotic lesions carry the AT_1 receptor.[13] Therefore, it is likely that angiotensin II plays an important role in the genesis of in-stent restenosis. We hypothesized that ACE-activity, either local or in plasma, is related to the occurrence of in-stent restenosis.
The aim of this study was to evaluate the correlation between plasma ACE-activity and the occurrence of in-stent restenosis. Therefore, we determined the amount of ACE in the original atherosclerotic plaque as well as the ACE-activity in plasma in a different group of patients.

METHODS

Plasma ACE
Consecutive patients scheduled to undergo a percutaneous coronary intervention with stent placement were enrolled. Exclusion criteria were prior PCI or CABG of the target lesion, current treatment for a malignancy or infection, use of immunosuppressive drugs, acute myocardial infarction and the use of an ACE-inhibitor and/or an angiotensin II type 1 receptor blocker.
Blood samples were drawn immediately before coronary angioplasty. Plasma samples were kept frozen at –80 °C until analysis. ACE-activity in plasma (35 times diluted) was measured according to the method of Cushman and Cheung,[14] using 10 minutes of incubation with 7 mmol/L of hippuryl-L-histidyl-L-leucine (HHL) at 37°C. The clinical investigators were blinded to the laboratory results.
Clinical follow-up was performed at 8 months by mailing a questionnaire to the patient or his relatives. Occurrence of angina pectoris, (target) PCI or CABG, myocardial infarction, and death were all recorded as adverse clinical events.

Tissue ACE
Tissue was collected from patients with significant, stable de-novo coronary lesions in the proximal left anterior descending artery or the circumflex artery. These patients did not use an ACE-inhibitor or an angiotensin II type 1 receptor blocker.
Percutaneous coronary atherectomy was performed using a pullback atherectomy catheter (Arrow int., Reading, PA, USA). In all patients, a biopsy was taken out of the culprit lesion.

Coronary angiography before and after the atherectomy confirmed that the sample was taken from the lesion. The samples were immediately frozen in liquid nitrogen and stored in a –80°C freezer. Following the atherectomy procedure, a stent was placed, and the minimal luminal diameter (MLD) was measured using quantitative coronary angiography (QCA)(CAAS-system, PIE-medical, Maastricht, the Netherlands). Of each specimen, 5 µm sections were mounted on glass slides. One slide was stained with haematoxylin & eosin (H&E) and another was used for immunohistochemistry with an anti-ACE antibody (mouse antibody; clone 9B9; Chemicon, Temecula, CA, USA). Sections of alveolar lung tissue and infarcted myocardium served as positive controls for anti-ACE staining.
The amount of ACE staining was applied by two independent investigators in order to evaluate the number of ACE-positive cells in each biopsy. The number of ACE-positive cells was expressed as a percentage of the total number of cells present in the biopsy as visualised in the adjacent H&E stained sections. The investigators were blinded to the patient characteristics. The patients were divided into two groups. Group 1: absent ACE staining, a few scattered cells or clusters <5 cells with positive ACE-staining; group 2: more than 5% of cells ACE-positive.
After 12 months, the patients underwent repeat angiography, during which QCA was performed and the MLD was measured. Luminal loss was defined as the difference between the MLD at baseline and after 12 months.

Both studies comply with the Declaration of Helsinki. The protocols were approved by the local ethics committees and informed consent of all patients was obtained.

Statistical analysis
Two-tailed T-test and Chi-square tests were performed to compare continuous and categorical variables, respectively. Differences were considered statistically significant at a level of $P<0.05$.

RESULTS

ACE-activity was measured in 178 patients who underwent a percutaneous coronary intervention with stent placement. The characteristics of these patients are summarised in table 1. In this group, 51 patients suffered from an adverse clinical event. As shown in figure 1, the ACE-activity in the plasma did not correlate with the occurrence of these adverse events (23.1 vs. 21.5 nM/ml/min; P=0.36).
Thirteen patients underwent an atherectomy. The biopsy tissue existed mainly of myxoid tissue with stellate shapes cells ('neointimal tissue') as has been described previously for in-stent restenotic lesions.[15] Anti-ACE staining revealed scattered positive cells in all but one lesion, but the total amount of positive staining was very low. Six patients had no (n=5) or only a few ACE-positive cells (n=1) in the biopsy of their original plaque. Of the other 7, the biopsy of six patients contained 5-50% ACE-positive cells, and in one case, more than

Table 1. Patient characteristics patient group A

	No adverse events (n=127)	Adverse events (n=51)	P
Age (years)	59.4	61.6	0.24
Male (%)	72.9	63.5	0.21
Smoking (%)	43.5	34.7	0.30
Hypertension (%)	20.6	23.1	0.72
Positive Family History (%)	49.1	46.7	0.78
Diabetes (%)	9.4	9.6	0.97
Total cholesterol (mmol/l)	5.86	6.22	0.14
Beta Blockers (%)	76.0	71.2	0.50
Calcium antagonists (%)	51.2	46.2	0.54
Nitrates (%)	59.7	61.5	0.82
Cholesterol lowering drugs (%)	41.9	42.3	0.96
Heparin (%)	14.0	9.6	0.43
Coumarin (%)	36.4	36.5	0.99

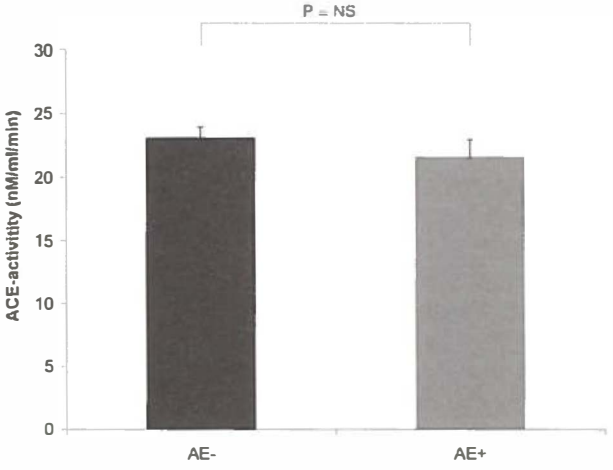

Figure 1. Plasma ACE-activity (nM/ml/min) in patients with (grey bars) or without (black bars) adverse clinical events in the 8 months following a percutaneous coronary intervention with stent placement.

Table 2. Patient characteristics patient group B

	No or few ACE-positive cells (n=6)	>5% ACE-positive cells (n=7)	P
Age (years)	52.7	53.3	0.88
Male (%)	100	85.7	1.00
Smoking (%)	50.0	28.6	0.59
Hypertension (%)	50.0	57.1	1.00
Positive Family History (%)	66.7	28.6	0.20
Diabetes (%)	0.0	0.0	1.00
Total cholesterol (mmol/l)	4.84	5.76	0.07
Beta Blockers (%)	83.3	71.4	1.00
Calcium antagonists (%)	50.0	71.4	0.59
Nitrates (%)	16.7	71.4	0.10
Cholesterol lowering drugs (%)	50.0	42.9	1.00
Heparin (%)	33.3	0.0	0.19
Coumarin (%)	0.0	0.0	1.00

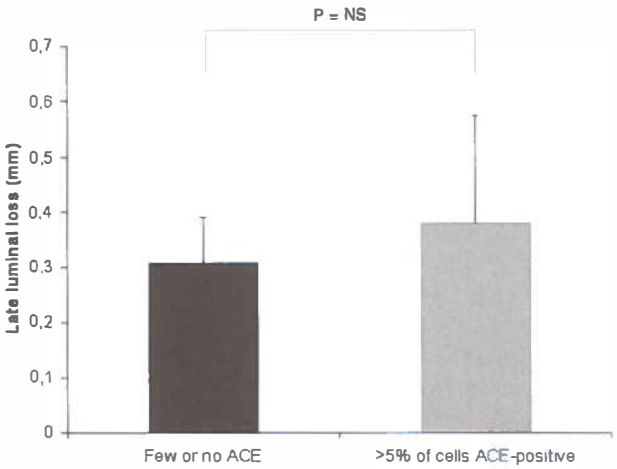

Figure 2. Luminal loss (determined with quantitative coronary angiography) 12 months after stent placement in patients with no or few ACE in their atherosclerotic plaque (black bars) or with >5% of ACE-positive cells in their plaque (grey bars).

50% of the cells were ACE-positive. There were no differences in the characteristics between the two groups (see table 2). At repeat angiography with QCA, no relation between the amount of ACE in the original plaque and late luminal loss could be found (0.31 vs. 0.38 mm; P=0.76)(see figure 2). Also the degree of in-stent restenosis did not differ (34.2 vs. 30.3%; P=0.59).

DISCUSSION

In-stent restenosis is a process that is mainly due to proliferation of vascular smooth muscle cells forming a variable amount of neointima within the stent.[11,16] In the first four weeks after stent placement, other processes like platelet deposition and inflammation at the strut sites play a role too, but after approximately one month, neither thrombi nor acute inflammatory cells are found in the neointima.[15,17] At that time, the plaque consists for 96% of vascular smooth muscle cells and proteoglycan matrix.[11] Chronic inflammation cells are only found at the strut sites.[16]

In a previous study, we demonstrated that VSMCs in the neointima of human in-stent restenotic lesions were mostly AT_1 receptor-positive.[13] The renin-angiotensin system is probably involved in the progress of in-stent restenosis as angiotensin II, the effector molecule of the renin-angiotensin system, is able to induce proliferation of VSMCs via its AT_1 receptor.[12,18,19] Indeed, the AT_1 receptor blocker valsartan was able to prevent in-stent restenosis in the Val-PREST study.[20]

Whether inhibition of ACE is effective in preventing in-stent restenosis is still unclear. Several studies reported that the ACE-inhibitor quinapril reduces in-stent intimal hyperplasia, late luminal loss and restenosis rate.[21-23] A recent retrospective study in 1598 patients suggested that ACE-inhibitors decrease late revascularisation after stent placement.[24] In the PARIS study, on the other hand, the ACE-inhibitor quinapril did not prevent in-stent restenosis in patients with the DD genotype of ACE.[25]

In this study, we demonstrated that plasma ACE-activity is not correlated with the incidence of adverse clinical events after stent placement. Moreover, in a small group of patients, we found that the content of ACE in the plaque during percutaneous coronary intervention did not predict the amount of in-stent restenosis after 12 months.

The absence of a correlation between ACE-activity and in-stent restenosis might have several causes. A limitation of our study is that we measured ACE-activity (both in the plasma as well as in the plaque) before stent placement. It is possible that the ACE-expression significantly changes as a reaction to the injury made by the balloon and/or the stent during PCI, as it does in animal models.[26] Another possibility is that ACE is not the rate-limiting step in the cascade of the renin-angiotensin system. This implicates that even in case of low ACE-activity, enough ACE is present to convert angiotensin I to angiotensin II. It remains controversial whether patients with the DD-allele of the ACE-polymorphism are especially at risk for in-stent restenosis, due to their higher ACE-activity. The D-allele

has been identified as a prognostic factor for in-stent restenosis in some studies,[27,28] but other larger studies did not confirm this.[29-31]
A third explanation for the absent correlation between ACE-activity and in-stent restenosis is that angiotensin I is not only converted to angiotensin II by ACE, but also by other enzymes. This hypothesis is supported by a study by Hojo et al., who showed that ACE-inhibitors had no inhibiting effect on the increased angiotensin production during PCI in humans.[32] Several studies have proven that in human vessels non-ACE converting enzymes, such as chymase and cathepsin G, could be involved in the production of angiotensin II.[33,34] In human coronary arteries, chymase is mainly found in mast cells in the adventitia,[35] and can therefore not be found in our biopsies. The number of chymase-positive cells is increased in atherosclerotic plaques in human aortas as well as in coronary arteries.[35,36] In a canine model, chymase is upregulated after balloon injury.[26] Notably, in the normal human heart chymase already appears to be the predominant enzyme for the angiotensin I conversion.[37] Since we only measured ACE-activity in our study, we cannot prove the importance of other enzymes in the genesis of in-stent restenosis.
In conclusion, we studied the relationship between the angiotensin converting enzyme (ACE) and the incidence of in-stent restenosis. We found that the ACE-activity in plasma at the time of the initial percutaneous coronary intervention was not correlated with the extent of in-stent restenosis. Furthermore, the presence of ACE in the initial plaque was also not associated with in-stent restenosis. Therefore, our study does not confirm that pre-procedural plasma and tissue ACE-activity are related to in-stent restenosis, although a relation between the renin-angiotensin system and in-stent restenosis remains undisputed.

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Chapter 5

Functional antagonism of different angiotensin II type 1 receptor blockers in human arteries

Lodewijk J. Wagenaar, Adriaan A. Voors, Hendrik Buikema, Azuwerus van Buiten, Rob H. Lübeck, Piet W. Boonstra, Dirk J. van Veldhuisen, Wiek H. van Gilst

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ABSTRACT

Objectives. To evaluate and compare the functional type and the degree of antagonism of the selective angiotensin II type 1 receptor blockers (ARB) losartan, EXP 3174 (the active metabolite of losartan), valsartan and candesartan in human internal mammary arteries.

Methods. Human internal mammary arteries were obtained as excess graft material during coronary bypass surgery. Vessels were prepared as rings and mounted in an organ bath in which vasoconstriction and –dilation can be measured. Concentration-response curves of angiotensin II-mediated vasoconstriction were measured in absence or presence of different concentrations of one of the ARBs.

Results. Losartan showed a rightward shift of the angiotensin II-mediated vasoconstriction, whereas addition of its metabolite EXP 3174 caused a decrease of the maximal effect of angiotensin II. Incubation with valsartan and candesartan also resulted in a decrease of the maximal effect. The inhibiting effects on the angiotensin II-mediated vasoconstriction by the highest concentration of EXP 3174, valsartan and candesartan did not differ significantly.

Conclusions. In human internal mammary arteries, losartan acts as a surmountable antagonist. On the other hand, EXP 3174, valsartan and candesartan demonstrate an insurmountable type of antagonism. Furthermore, the inhibiting effects of EXP 3174, valsartan and candesartan in our study are equal in the highest concentrations.

Since the first description of losartan in 1988, several selective Angiotensin II type 1 Receptor Blockers (ARB) have been developed.[1] It appears that not all ARBs adhere to the classical and most common competitive receptor antagonism. The type of antagonism seems to be species-dependent as well as vessel-dependent.[2-8] In rabbits, for instance, losartan antagonises angiotensin II (Ang II)-induced contractions insurmountably in renal arteries,[6] but causes surmountable antagonism in aortic rings.[2]
Few studies have been published about the types of antagonism of ARBs in human arteries. In one study, the effect of losartan, EXP 3174 and candesartan on Ang II-induced vasoconstriction in resistance vessels has been examined.[9] Two other studies describe the antagonistic effects of losartan in respectively human gastroepiploic arteries and human coronary arteries.[10,11] Both studies demonstrate an insurmountable type of antagonism. However, a comparative study on the effects of different ARBs in human conductance arteries has not been performed.

The aim of this study is to evaluate and compare the functional types of antagonism of the angiotensin II type 1 receptor blockers losartan, EXP 3174 (the active metabolite of losartan) valsartan and candesartan in human internal mammary arteries.

METHODS

Human vessels
Segments of the human internal mammary arteries were collected during coronary bypass surgery as excess graft material in the University Hospital of Groningen, the Netherlands. The segments were placed in a buffer solution (Krebs) of the following composition (mM): NaCl (120.4), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), glucose (11.5), and $NaHCO_3$ (25.0). The segments were processed within 24 hours after removal. The study conforms with the principles outlined in the Declaration of Helsinki.

Functional measurements
The vessels were cleaned of surrounding tissue and cut into several rings (2 mm). The rings were then mounted in 15-ml organ baths, containing the aerated Krebs solution at 37 °C and connected to an isotonic displacement transducer.
In every single experiment, we studied several arterial rings from one donor in parallel fashion. We usually obtained an arterial segment long enough to produce 7-8 rings for different organ baths. Two of these rings were always used to obtain control responses (incubated with vehicle) in each experiment, while the remaining number of (n=5-6) rings of the same vessel segment were used to test the effect of different doses of one of the ARBs (i.e. each ring incubated with a different dose of a particular ARB).
Rings were given a preload of 14 mN and were allowed to equilibrate for at least 60 minutes, during which regular washing periods were performed.[12] Rings were then checked for viability

by repeated stimulation with 10 mM phenylephrine, followed by washing and restabilisation. Rings that failed to reach a contractile response to phenylephrine of at least 100 micrometer displacement were not included in the experiment. Rings were then incubated with the NO-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, 0.1 μM) to prevent any confounding effect due to potential interindividual differences in the basal release of nitric oxide opposing vasoconstriction. After 10 minutes one of the ARBs in different concentrations (0.1 nM to 100 nM) or solvent (NaCl 0.9%) was added. Most experiments consisted of 8 rings from one artery. Two of these rings always served as control.
Twenty minutes after the preincubation with an ARB, cumulative concentrations of angiotensin II (Ang II, 0.1 nM to 1 mM) were administered and contraction displacement was assessed. Afterwards, the rings were stimulated to a maximum by a bolus of 10 mM phenylephrine to demonstrate intact vasoconstriction.
Vessels in which the maximum response to Ang II in the control ring in absence of an ARB did not reach at least 20% of the response to phenylephrine in the same ring, were regarded not evaluable and excluded for further analysis.

Drugs
Losartan was a gift from Merck Sharpe & Dohme Research Laboratories (Rahway, NJ, USA), Valsartan was a gift of Novartis Pharma AG (Basle, Switzerland), Candesartan was a gift from Astra Pharmaceutica BV (Zoetermeer, The Netherlands). Angiotensin II was obtained from CIBA-Geigy ltd. (Basle, Switzerland). L-NMMA and phenylephrine were obtained from Sigma (St Louis, MO, USA). The drugs were dissolved in ethanol (EXP 3174) or saline (others) and freshly prepared daily from stock solutions.

Statistical methods
Contractions to Ang II were related to the maximum contraction to phenylephrine in individual rings, as to avoid non-specific differences between rings from the same patients. Subsequently the reaction to Ang II was expressed as percentage of the maximal relative contraction to Ang II in the control ring of the same vessel to control for interindividual differences in responsiveness to Ang II.

Curve fits were made and the EC50s were estimated by using the curve fitting option of SigmaPlot 3.0 for Windows. Comparisons between the complete concentration-response curves were made by repeated measures analysis of variance. Calculations were performed using the GLM procedure of SAS for Windows 6.12. Design was made according to recommendations by Ludbrook[13]. Since the sample number in each group was not equal, the Greenhouse-Geisser adjustment was made for multisample asphericity. A probability level of <0.05 was considered significant.

RESULTS

The ARBs did not have any vasomotor effects on the basal tone (data not shown). Full concentration-response curves for angiotensin-induced contraction in the presence of losartan, EXP 3174, valsartan and candesartan are shown in figures 1a to d (n=7-10 for each curve).
All ARBs except losartan show a concentration-dependent decrease of the maximal response, indicating an insurmountable type of antagonism. We cannot exclude a possible rightward shift in EXP 3174 (fig. 1b) and valsartan (fig. 1c). Losartan shows only a concentration-dependent rightward shift, indicating a surmountable type of antagonism (fig. 1a).
The losartan curve only differs significantly from the control curve at its highest concentration level (100 nM) (fig. 1a). The other curves, on the other hand, differ from the control curves at 1 nM and higher concentrations.

Equal concentrations of different ARBs have been compared as described in the statistical methods. At the highest concentration (100 nM) the angiotensin II inhibiting effects of candesartan, EXP 3174 and valsartan are comparable, whereas the effect of losartan differs significantly from the other curves at the highest concentration.

DISCUSSION

Receptor antagonists can be described as competitive vs. non-competitive, or as surmountable vs. insurmountable. These terms are frequently confused. Surmountable antagonism means that the inhibition caused by the antagonist can be surmounted, i.e. overcome with increasing concentrations of an agonist, whereas in the presence of a insurmountable antagonist an agonist cannot reach its maximal effect, irrespective of its concentration. Competitive antagonism, on the other hand, means that the agonist and the antagonist compete for binding to the receptor. Non-competitive antagonism may be regarded as an irreversible binding to the receptor.[14,15] The insurmountable ARBs may appear to be non-competitive antagonists. However, these antagonists do compete at the site where angiotensin interacts with the receptor, as has been shown for candesartan and EXP 3174.[16,17] The insurmountable characteristics of these ARBs can be explained by their slow dissociation from the angiotensin II type 1 receptor.[18,19]
In our study design, the functional antagonism of ARBs on Ang II-induced vasoconstriction has been evaluated. Therefore, we can determine whether the antagonist is surmountable or not, but we cannot distinguish between competitive and non-competitive antagonism.[20]

Figures 1a-d. Concentration-response curves of Angiotensin II (Ang II)-induced vasoconstriction in presence of losartan (fig. 1a), EXP 3174 (fig. 1b) valsartan (fig. 1c) and candesartan (fig. 1d). Values are mean ± SEM.
*‡: P<0.05 vs. control; *: P<0.0001 vs. control*

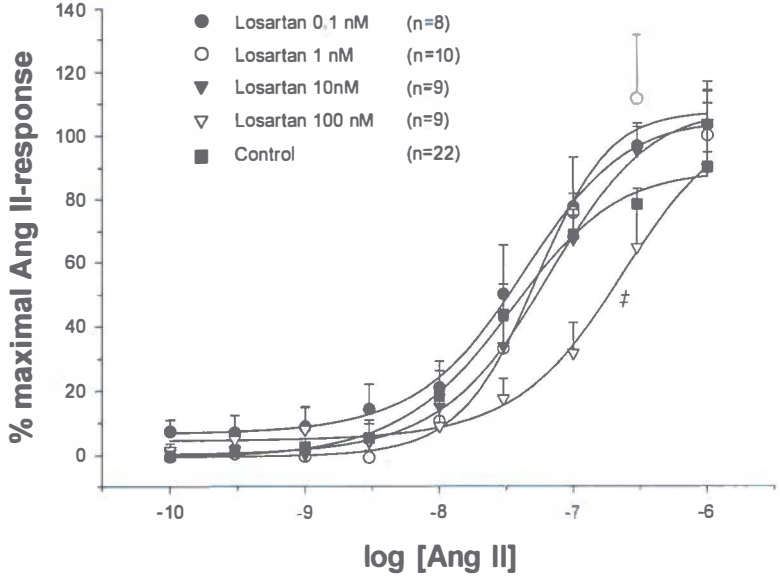

Figure 1a

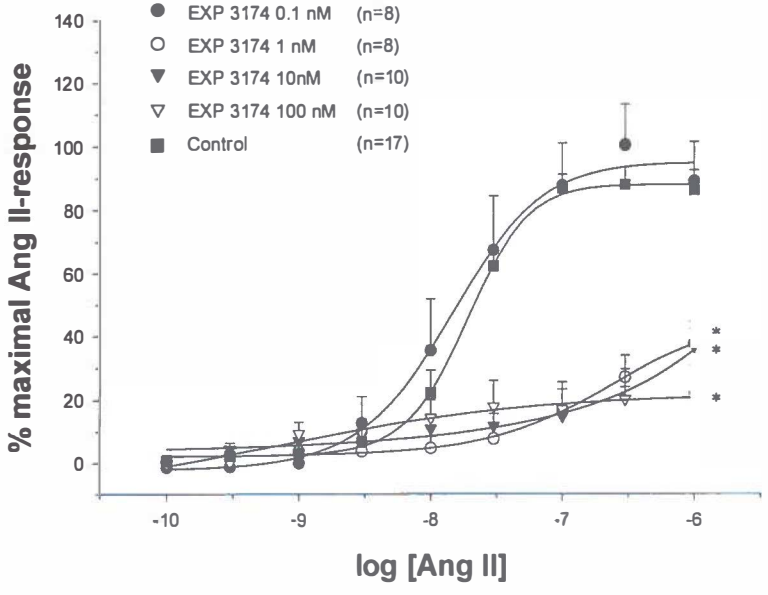

Figure 1b

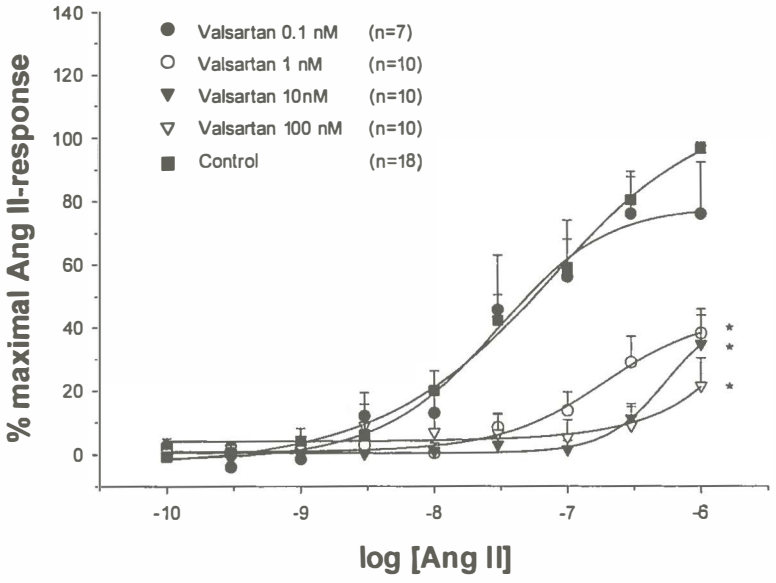

Figure 1c

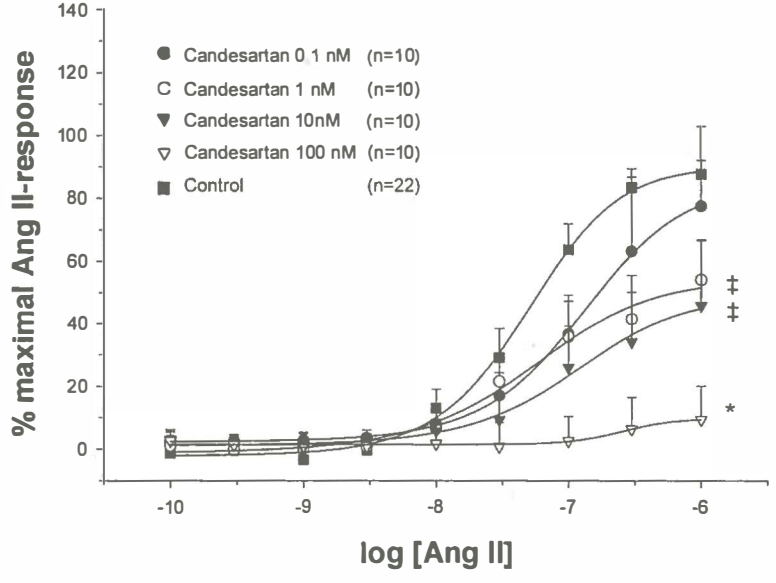

Figure 1d

In our human study losartan appears to be a surmountable antagonist in internal mammary arteries. This is consistent with experiments performed in isolated rabbit aortic strips and in human gastroepiploic arteries,[4,10,21-23] but inconsistent with other human studies. In human gluteal subcutaneous resistance arteries, as well as in coronary arteries, losartan seems to inhibit Ang II induced vasoconstriction insurmountably.[9,11] Also, in an in-vivo model of human forearm blood flow, losartan had an insurmountable kind of antagonism on the Ang II-mediated decrease in flow.[7] In the last experiment, measurements were made 5 minutes after infusion of losartan, so the effect could be mediated partially by its active metabolite EXP 3174.[24] In isolated rabbit femoral arteries too, losartan had an insurmountable kind of antagonism on Ang II-induced contraction.[6]

EXP 3174, the active metabolite of losartan, inhibits Ang II-induced vasoconstriction insurmountably, as it does in isolated rabbit aortas[3] and human gluteal subcutaneous resistance arteries.[9] Since we cannot exclude a rightward shift on top of the decrease of the maximal response to angiotensin II, EXP 3174 might be a combination of a surmountable and an insurmountable antagonist.

Valsartan is sometimes presented as a surmountable antagonist,[25] but in isolated rabbit aortic strips valsartan appears to behave as a combination of a surmountable inhibitor (in lower concentrations) and a insurmountable inhibitor (in higher concentrations) of Ang II-mediated vasoconstriction.[5] In the present study valsartan behaves as an insurmountable antagonist, although this does not exclude the above-mentioned properties of a combined surmountable and insurmountable inhibitor.

Candesartan cilexetil is metabolised completely to candesartan during absorption in the gastrointestinal tract.[26] Candesartan can be characterised as an insurmountable antagonist in human internal mammary arteries. This is confirmed by studies in various species and vessels, including isolated rabbit aortas and human subcutaneous resistance arteries.[4,8,9,17]

In our experiments the blocking effects of the highest concentrations (100 nM) of the antagonists did not differ significantly between candesartan and valsartan. Although the blocking effect of the highest concentration of losartan was different, its antihypertensive effect is at least partially mediated via its active metabolite EXP 3174, whose concentration-response curve did not differ from the candesartan and valsartan curves.

Several studies indicated a combination of both surmountable and insurmountable antagonism of some ARBs. This can be explained using the 2-state model.[15,27] In this model an equilibrium between an active and an inactive state of a receptor is proposed. In the active state, the receptor can mediate a biological response after binding to an agonist. If the receptor is in its inactive state, binding to an agonist will not led to a biological response. The ratio between these states determines the maximum achievable response. An antagonist that has an equal affinity for both states of the receptor will not change the

equilibrium between the two states, and therefore act as a surmountable antagonist. Insurmountable antagonists on the other hand are believed to have a higher affinity for the inactive state of the receptor, thereby causing a change in the equilibrium towards the inactive state. In this case fewer active receptors are available to bind to angiotensin II, thus causing a reduction of the maximum achievable response.

Study limitations

The results of this study are more difficult to interpret than studies in rats or rabbits, because responses in human arteries are more variable. This may be caused by interindividual differences in humans and by differences during surgery. Furthermore, we chose to perform a protocol with fixed concentrations of the antagonists and of angiotensin II. This may have lead to incomplete concentration-response curves, and in the case of EXP 3174, to a gap between different curves.

In our protocol, the antagonists were incubated for 20 minutes before the concentration-response curve with angiotensin II was started. We cannot be sure that an equilibrium between the different receptor states has been achieved at that moment. But as, for instance, the highest concentration of candesartan inhibits the angiotensin II-mediated vasoconstriction almost completely, we assume that the equilibrium has been reached in the organ baths at that moment.

CONCLUSIONS

This study was designed to evaluate and compare the degree and type of antagonism of angiotensin II type 1 receptor antagonists in human internal mammary arteries. In our experiment, losartan turned out to be a surmountable antagonist, whereas its active metabolite EXP 3174, candesartan and valsartan are insurmountable. This is in accordance with studies performed in rabbit aorta strips, the model that is mostly used to evaluate types of antagonism of ARBs.

The degree of blockade of candesartan, EXP 3174 and valsartan in the present model appears to be equal in the maximum concentration used.

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Chapter 6

Improvement of endothelial dysfunction in experimental heart failure by chronic RAAS-blockade: ACE-inhibition or AT_1 receptor blockade?

Lodewijk J. Wagenaar, Hendrik Buikema,
Yigal M. Pinto, Wiek H. van Gilst

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ABSTRACT

Chronic heart failure (CHF) is associated with endothelial dysfunction. Activation of the renin-angiotensin-aldosterone system (RAAS) is believed to be important in the deterioration of endothelial dysfunction in CHF through stimulation of oxidative stress. Whereas ACE-inhibitors (ACEi) improve endothelial function in CHF, the effect of angiotensin receptor blockers (ARB) is less well established. Therefore we compared the effect of the ACEi lisinopril versus the ARB candesartan on endothelial dysfunction in a rat model of CHF.
CHF was induced by myocardial infarction (MI) after coronary ligation. Two weeks after MI, daily treatment with lisinopril (2 mg/kg) or candesartan (1.5 mg/kg) was started. After 13 weeks rats were sacrificed and the endothelial function was determined by measuring acetylcholine (ACh)-induced vasodilation in aortic rings, with selective presence of the NOS-inhibitor L-NMMA to determine NO-contribution.
ACh-induced vasodilation was attenuated in untreated MI (-50%) compared with control rats. This was in part due to impaired NO-contribution (-49%). Lisinopril and candesartan fully normalised ACh-induced dilation, including the part mediated by NO.
Chronic RAAS-blockade with LIS and CAN normalised endothelial function in CHF in a comparable way. The effect of both treatments included the increase of the NO-mediated dilation, further indicating the important role of oxidative stress in the relation between the RAAS and endothelial dysfunction in CHF.

Experimentally in animal models of chronic heart failure (CHF)[1-3] as well as clinically in heart failure patients,[4,5] endothelial dysfunction has been identified as the impaired dilatory response to acetylcholine in a setting of preserved responsiveness to vasodilators such as nitroprusside. This endothelial dysfunction leading to abnormal vasomotor control may give rise to increased peripheral vascular resistance, a hallmark of CHF. As such, endothelial dysfunction is regarded to be one of the underlying and contributing factors for the progressive nature of chronic heart failure (CHF).[5] Consequently, prevention or reversal of the endothelial dysfunction may be considered an important target for pharmacological intervention in CHF.

The chronically activated (tissue) RAAS is believed to importantly contribute to the deterioration of cardiovascular function,[6,7] including in CHF. Angiotensin II (Ang II), the effector molecule of the RAAS, has shown to be an important stimulus for increased oxidative stress through vascular superoxide production via membrane NADH/NADPH oxidase activation.[8] Hence, reactive oxygen radicals may importantly contribute to endothelial dysfunction through inactivation of endothelium-derived nitric oxide (NO).[9] Recent experimental findings suggest that heart failure is associated with increased oxidative stress, and that this may underlie endothelial dysfunction in CHF through increased vascular superoxide production.[10]

Ang II is first cleaved by ACE (amongst others) from angiotensin I (Ang I) before it exerts its effect through stimulation of the angiotensin II type 1 receptor (AT_1r).[11] Using the rat coronary ligation/myocardial infarction (MI) model of CHF, several investigators reported intervention in the RAAS through chronic ACE-inhibition to improve or even restore the response to acetylcholine.[2,3,12] This is in line with the above-mentioned idea that the Ang II-mediated increase in oxidative stress underlying impaired NO-activity in endothelial dysfunction in CHF is counteracted by ACE-inhibitors through prevention of ACE-mediated Ang II-formation. If true, one would predict that similar effects might be obtained by preventing the effect of Ang II in increasing oxidative stress through blockade of the AT_1 receptor (AT_1r). In support of this are reports describing comparable beneficial effects of chronic RAAS-blockade after ACE-inhibition and AT_1 receptor antagonism on other parameters such as left ventricular ejection fraction, interstitial collagen fraction, myocardial hypertrophy and survival.[13-15] However, similarities or discrepancies of chronic treatment with an ACE-inhibitor (ACEi) compared to an AT_1-receptor blocker (ARB) regarding their effects on endothelial dysfunction in CHF has been less well established.

To address this item, therefore, MI-induced heart failure rats were compared for improvement of aortic endothelial dysfunction after chronic RAAS-blockade with either the ACEi lisinopril (LIS) or the ARB candesartan (CAN). Endothelial function was assessed as the dilatory response to acetylcholine (ACh), and the NOS-inhibitor L-NMMA was used to determine the contribution of NO in the response.

METHODS

Myocardial infarction model

All procedures were reviewed and approved by the Animal Research Committee at the University of Groningen. Normotensive male Wistar rats (250-300 g; Harlan, Zeist, the Netherlands) were housed group-wise at the Central Animal Laboratory, University of Groningen (the Netherlands). They had free access to food and water. At the time of the operation anaesthesia was induced with isoflurane (2.0 – 2.5% in oxygen), after which rats were intubated and mechanically ventilated with this gas-mixture (Amsterdam Infant Ventilator, Hook/Loos, Schiedam, the Netherlands). MI was induced by direct coronary ligation as described before.[16] Briefly, a left-sided thoracotomy was performed and the anterior descending coronary artery occluded with a 6-0 silk suture 1-2 mm after the bifurcation. Care was given to obtain a blanching of the ligature to confirm MI. If necessary the procedure was repeated by placement of a second or third ligature. Thereafter the thorax was closed and as soon as spontaneous respiration was sufficient, the rats were extubated and were allowed to recover under a heated lamp.

Stratification and treatment

One week after surgical procedures, surviving rats were anaesthetised and subjected to ECG-measurements, based on which individual rats with ECG-evidence (averaged QT-interval prolongation in three pre-cordial leads) for MI were assigned to treatment with LIS, CAN, or no-treatment. Rats with minor or no ECG-evidence for MI were additionally allocated to a no-treatment regimen to function as a control group, giving rise to 4 experimental groups in total. As described previously,[17] ECG-stratification was used for a balanced distribution of MI-rats with different MI-size over the experimental MI-groups only, and in all cases group stratification was checked post-mortem by quantitative left ventricular histopathology.

Rats allocated to one of the two active treatment regimens received either lisinopril or candesartan cilexetil mixed with the chow (Hope Farms, Woerden, The Netherlands). The remaining rats served as non-treated controls. Based on drug analysis of the food (Dr. P. Morsing, AstraZeneca, Mölndal, Sweden) together with the assessment of food-intake and body weight, the average daily dose of lisinopril and candesartan cilexetil was approximately 2 mg/kg and 1.5 mg/kg, respectively. To avoid a potential effect of early pharmacological intervention on infarct-size and wound healing,[3,18] treatment was started 2 weeks after induction of MI (i.e. established MI). Treatment lasted for 11-12 weeks and animals were sacrificed at 13-14 weeks after initial surgical procedures.

Sacrifice

The rats were anaesthetised with isoflurane in 2% oxygen. The right carotid artery was catheterised with a polyethylene catheter filled with 0.9% saline with heparin (5.000 U/L). The carotid catheter was advanced into the aorta for recording of the aortic blood pressure

(Stratham 23 Db, Gould Instruments, Cleveland, Ohio, USA).
Subsequently, heparin (5.000 IU kg^{-1}) was administered via the tail vein. Then first the hearts were excised and rapidly arrested in icy-cold NaCl, and mounted in an organ perfusion set-up. Retrograde perfusion of the aorta at 38 °C, essentially by the Langendorff method, was achieved immediately. The hearts started to beat spontaneously and after equilibration for 10 minutes baseline measurements were performed on LV pressure, contractility, relaxation, heart rate and coronary flow, as described in detail elsewhere.[16,19] Thereafter, hearts were arrested in diastole in KCl (2 mol/L) and weighed. To determine the infarct size the scar to total LV circumference ratio was measured as described in detail elsewhere.[18] Rats that were assigned to one of the MI-groups but which appeared to have an infarct-size <20% were excluded from analysis.
After excision of the heart the thoracic descending aorta was removed and placed in a buffer solution (Krebs) of the following solution (mM): NaCl (120.4), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), glucose (11.5), and $NaHCO_3$ (25.0).

Organ bath studies with isolated aortic rings
The aorta was cleaned of adhering tissue and rings of 2 mm in length were cut with a sharp razor blade. Rings were then mounted in an organ bath containing the above-mentioned Krebs buffer at 37 °C, aerated with 95% O_2 and 5% CO_2, and indomethacin (10 mM) to avoid potential interference of vasoactive prostanoids. Rings were connected to an isotonic displacement transducer at a preload of 14 mN and were allowed to equilibrate for at least 60 minutes, during which regular washing periods were performed.[20] Rings were then checked for viability by repeated stimulation with 1 mM phenylephrine (PE).
After washing and restabilisation, one subset of rings was precontracted with 1 mM PE. The response to increasing doses of the endothelium-dependent vasodilator ACh (10 nM to 10 mM), in presence or absence of the NO-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, 100 mM), and to the endothelium-independent dilator nitroglycerin (NTG, 1 nM to 10 mM), in absence of L-NMMA only, was determined. In another set of rings, the contractile response to increasing concentrations of Ang II (0.1 nM to 1 µM) was measured in the presence of L-NMMA.[21] Subsequently these rings were contracted to their maximum by a bolus of PE (1 mM).

Calculations and statistical analysis
Vasodilator responses to ACh and NTG and constrictor responses to Ang II were expressed as a percentage of PE-induced pre- and post-contraction, respectively, before individual concentration-response curves were generated and the Area Under each individual Curve (AUC) was determined (SigmaPlot scientific graphing software package, Jandell Scientific). The AUC was used to represent the individual response-size to a given agonist in a given condition and, where appropriate, to calculate the difference in response-size between different conditions (i.e. difference in response-size in presence of indomethacin versus indomethacin plus L-NMMA for ACh). In this way, employing the AUC provided us with a simple (and straightforward) method for estimation and analysis of the L-NMMA-sensitive

(hence, NO-mediated) and –resistant (hence, endothelial derived hyperpolarising factor (EDHF) or other-mediated) part of the response to ACh. For reasons of consistency, the AUC was also used to calculate and present the average response-size for each group, as well as for subsequent analysis of differences among groups.
Unless stated otherwise, all data are expressed as mean±s.e.mean. The four experimental groups were compared among each other using oneway-analysis of variances (ANOVA) in combination with Dunnet post-hoc analysis for multiple comparison, using no-MI as control category (SPSS for windows Standard Version 8.0). In addition, MI-groups were compared among each other using ANOVA in combination with Dunnet post-hoc analysis using untreated MI as control category. Differences were considered significant at a level of $p<0.05$.

RESULTS

Rats that survived all surgical procedures, had an infarct-size >20% in case of MI-groups, and which successfully underwent all experimental studies were included for analysis. Final group-sizes based on which the results are presented are n=8 for no-MI, n=11 for MI, n=8 for LIS and n=6 for CAN.

Rat characteristics
Mean infarct-size was evenly balanced between the 3 experimental MI-groups, and the rats in these groups all showed clear evidence of left ventricular (LV-)dysfunction, compared to rats with no-MI (Table 1). We have previously shown that LV pressure and dP/dT (i.e. contractility and relaxation) are significantly decreased and become progressively attenuated in rat with MI-induced heart failure.[16] Concurrent hemodynamic abnormalities in this MI-model of heart failure are related to increased cardiac weight and infarct-size.[22,23] In the present study, untreated MI-rats displaying an infarct-size of 34% on average were characterised by LV-dysfunction and increased heart and lung weights (Table 1). And also baseline coronary flow – which is co-determined by intact coronary endothelial function – was significantly decreased in untreated MI. In contrast, LIS and CAN prevented the increase in heart and lung weight, thus suggesting that both treatment regimens effectively prevented the progression of LV-dysfunction to CHF. Moreover, baseline coronary flow was significantly larger after LIS and CAN, thus suggesting that coronary endothelial function was preserved after both treatments.

Ang II–induced vasoconstriction
In the present study, Ang II–induced vasoconstriction (calculated and presented as AUC in Figure 1; see also methods) was significantly increased after treatment with LIS, as compared to no-treatment, while after CAN it was virtually abolished. The differences in the AUC were a result of alterations in the maximum effect to Ang II rather than a left/rightward shift of the concentration-response curve (data not shown). We have previously shown that

Table 1. Infarct-size, rat characteristics and baseline cardiac function.

	no-MI (n=8)	MI (n=11)	LIS (n=8)	CAN (n=6)
MI-size (% LV)	<5	34±3*	36±4*	32±5*
Body weight (g)	502±14	489±20	397±17*#	457±9
Aortic BP (mm Hg)	110±7	99±4	97±5	96±3
Lung : BW (mg/g)	3.3±0.3	4.9±0.8*	4.0±0.1	3.5±0.1
Heart : BW (mg/g)	4.0±0.2	5.6±0.5*	4.8±0.3	4.6±0.2
LV Pressure (mm Hg)	104±3	71±6*	74±6*	78±6*
Contractility (mm Hg/sec)	4104±119	3187±228*	2887±247*	2915±297*
Relaxation (mm Hg/sec)	3514±163	1859±149*	2009±147*	2116±166*
Heart Rate (beats/min)	295±21	267±9*	294±10*	285±20*
Coronary Flow (ml/min/g)	14.4±1.1	11.5±0.7*	14.6±0.6#	15.4±1.7#

**: p<0,05 vs no-MI*
#: p<0,05 vs MI
MI: myocardial infarct
BP: blood pressure
BW: body weight
LV: left ventricle

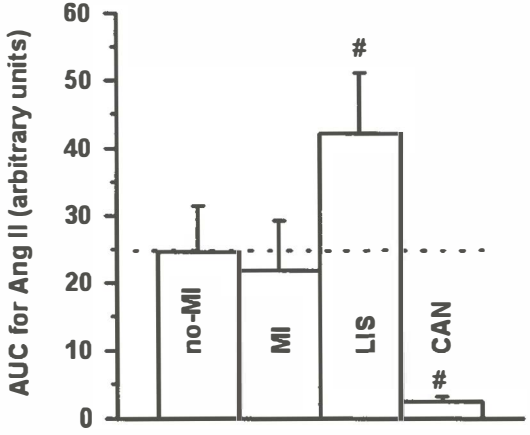

Figure1: Response-size to Ang II, displayed as area under the curve (AUC), in isolated aortic rings from untreated rats with no myocardial infarction (no-MI), untreated rats with MI (MI) and MI-rats treated with lisinopril (LIS) or candesartan (CAN). The broken lines represent the level of the no-MI group.
Data are mean±s.e.mean. # indicates p<0.05 versus MI.

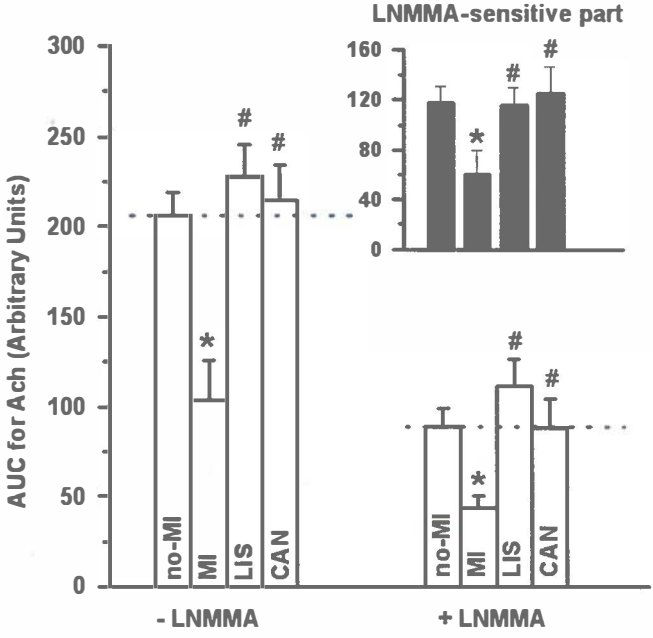

Figure 2: Response-size to ACh, displayed as area under the curve (AUC), in isolated aortic rings from untreated rats with no myocardial infarction (no-MI), untreated rats with MI (MI) and MI-rats treated with lisinopril (LIS) or candesartan (CAN). ACh-mediated vasodilation was studied in the absence or presence of L-NMMA, as indicated. The difference in response between the two conditions representing the L-NMMA-sensitive part of the vasodilation is shown in the graph on the right-side top. The groups from left to right are the same as in the graph on the right-side bottom.
The broken lines represent the level of the no-MI group. Data are mean±s.e.mean.
** indcates p<0.05 versus no-MI. # indicates p<0.05 versus MI.*

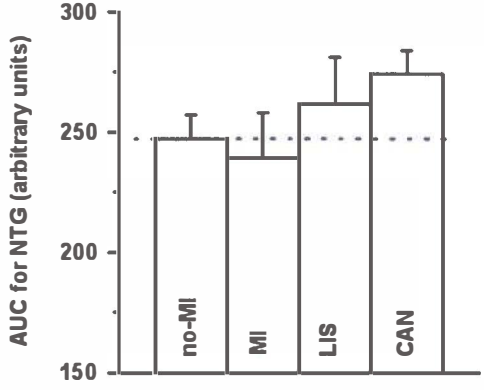

Figure 3: Response-size to NTG, displayed as area under the curve (AUC), in isolated aortic rings from untreated rats with no myocardial infarction (no-MI), untreated rats with MI (MI) and MI-rats treated with lisinopril (LIS) or candesartan (CAN). The broken lines represent the level of the no-MI group. Data are mean±s.e.mean.

effective – biochemically assessed - inhibition of vascular ACE after chronic oral intake of an ACEi results in an increased response to Ang II (possibly due to AT_1 receptor upregulation) in isolated rings in vitro.[21] Taken together, these data may provide functional evidence for effective RAAS-inhibition at vascular level after chronic oral LIS and CAN in the present study.

ACh-induced vasodilation

In the present study, the response-size of ACh was markedly impaired in the group of untreated MI-rats at 13 weeks post-MI (-50% on average), as compared to those with no-MI (Figure 2). The decrease of the AUC in the MI-rats was due to a decrease in the maximum response to ACh rather than differences in sensitivity to ACh (data not shown). This is consistent with previous studies suggesting progressive endothelial dysfunction in the present MI-model, first detectable in the aorta as the impaired response to ACh at approximately 8 weeks post-MI.[1,2,3,12] Inhibition of NOS using L-NMMA significantly reduced the response to ACh ($P<0.001$, Figure 2) in the present study, indicating the important involvement of NO. However, the L-NMMA-sensitive part of the dilator response was significantly smaller in untreated MI-rats (-49% on average), indicating a decreased NO-contribution compared to control rats with no-MI. Moreover, the part of the response to ACh resistant to L-NMMA (hence, and indomethacin) was also significantly decreased in untreated MI (-49% on average), pointing at a decreased contribution of a non-prostanoid/non-NO vasodilator substance as well. Treatment with LIS and CAN both fully restored the response the ACh, including the part mediated by NO.

NTG-induced vasodilation

The intact endothelium-independent response to NTG rules out that a potential impairment at the level of vascular smooth muscle reactivity to NO might have accounted for the above attenuated response to ACh in untreated MI (Figure 3). Furthermore, LIS and CAN did not significantly alter the response to NTG, thereby excluding the possibility that their beneficial effects on ACh-induced dilatation were in fact due to increased reactivity to exogenous NO.

DISCUSSION

We compared the vasoprotective effect of the ACEi lisinopril versus the ARB candesartan on the endothelial function in a rat model of experimental CHF. We found that candesartan and lisinopril normalised endothelial function in a similar fashion.

Endothelial function in the present study was assessed by the dilative response to ACh in isolated aortic segments. Several endothelium-derived substances may be involved in this response, including NO, vasoactive prostanoids, and EDHF.

Stimulation of endothelial cells with ACh leads to increased intracellular calcium concentrations, resulting amongst others in activation of NO synthase (NOS) and stimulated NO-production.[24] Recent findings suggest a marked downregulation of the NO-contribution to ACh-induced dilation in the rat MI-model of CHF.[25] This attenuated contribution might be caused by a decreased NO-formation, resulting from changes at the level of the receptor for ACh and/or at the level of signalling pathway leading to impaired NOS-activation. On the other hand, a raised NO-breakdown by increased stimulation of NO-inactivating pathways might also be involved. Oxygen radicals, such as superoxide (O_2^-) can inactivate NO.[9] Superoxide, produced by aortic NADPH, is reported to impair the ACh-induced vasodilation in the rat MI-model of CHF.[10] Other studies confirmed the relation between oxidative stress and CHF.[9] Indeed, superoxide dismutase was able to restore the impaired ACh-mediated vasodilation in CHF rats.[10]

CHF is associated with an activated RAAS in the present rat MI-model as well as in humans.[16] Ang II - the effector molecule of the RAAS - is able to induce the production of superoxide, leading to increased oxidative stress. In addition, ACE - the enzyme responsible for conversion of Ang I to Ang II - facilitates the breakdown of bradykinin, a potent vasodilator and anti-ischemic substance.[26] So blockade of the RAAS is expected to be an effective pharmacological option. Indeed ACE-inhibitors as well as ARBs have proven to be an effective treatment in preventing signs of left ventricular dysfunction and heart failure in the rat model and in humans.[13-15,27-31] Moreover, ACE-inhibition has proven to be able to normalise the response to ACh in heart failure.[2,3,12]
The effect of blockade of the AT_1 receptor by an ARB on the endothelial function is less well established. Since ARBs inhibit the effects of Ang II by blocking the AT_1r, they exert their effect by only attenuating the processes induced by Ang II, such as the increased oxidative stress. Unlike ACE-inhibition, therefore, Ang II receptor blockade will not lead to increased availability of bradykinin.

In our study, the attenuation of the vasodilative response to ACh in rats with untreated chronic heart failure could be fully restored by chronic RAAS-blockade. Moreover, ACE-inhibition (LIS) and AT_1r blockade (CAN) were equally effective. This might indicate that the effects of ACE-inhibition on rats with CHF are mediated mainly by diminishing the conversion of Ang I to Ang II, and not so much by increasing the availability of bradykinin. The attenuation of the vasodilative response to ACh is in part due to the decreased NO-activity in the aortic wall, since the L-NMMA-sensitive part of the vasodilation was decreased in the MI-group. Treatment with LIS as well as with CAN increased the NO-dependent vasodilation to the level of the rats with no MI. These results ground the idea that the improvement of endothelial function by LIS and by CAN is in part due to decrease of Ang II-induced oxidative stress.

Previous studies showed the limited role of vasoactive prostanoids in the aortic response to ACh in normal rats as well as in rats with heart failure.[1,32] In our study design, the rat aortas

were incubated with indomethacin to fully exclude the possible influence of prostanoids on the endothelium.
Despite the presence of LNMMA and indomethacin the aorta was still able to dilate in response to ACh. This part of the vasodilation was attenuated too in the non-treated MI-group, and both LIS and CAN restored this part of the dilation to the level of the no-MI group. EDHF is the most likely candidate for this non-NO, non-prostanoid-mediated vasodilation, but we cannot establish the nature of this part of the vasodilation, since we did not collect evidence for hyperpolarisation of the vascular smooth muscle cells.

In conclusion, chronic RAAS-blockade after ACE-inhibition with lisinopril or angiotensin II receptor blockade with candesartan similarly normalised endothelial function in our rat model of MI-induced chronic heart failure. This improvement includes a restoration of the NO-mediated dilation after RAAS-blockade in our model, suggesting an important role of oxidative stress in the relation between the RAAS and the endothelial dysfunction in CHF.

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Chapter 7

Summary and future directions

The circulating renin-angiotensin system (RAS) is a hormonal system, involved in the maintenance of a constant blood circulation. Angiotensin II, the main effector molecule of the RAS, is involved in many tissue bound processes that are important in vascular and cardiac function, including vasoconstriction, growth of vascular smooth muscle cells and of fibrotic tissue. Several risk factors can lead to an activated RAS, thereby inducing pathological conditions resulting in cardiovascular diseases, such as hypertension, atherosclerosis and heart failure.

The circulating renin-angiotensin system consists of a cascade of enzymes, starting with angiotensinogen, which is produced in the liver. Under influence of renin (produced in the kidney), angiotensinogen is converted to the still inactive angiotensin I. By cleaving the last two amino acids of angiotensin I, angiotensin II is created. This cleavage can be induced by several enzymes, among which the angiotensin converting enzyme (ACE) is the most important. Angiotensin II exerts its effects by stimulating its receptors, the angiotensin type 1 (AT_1) and type 2 (AT_2) receptors.
Apart from this circulating system, many tissues are capable to generate angiotensin II locally as a paracrine hormone.

Chapter 1 gives an overview of the role of the angiotensin receptors in the cardiovascular system. After a short summary of the steps that led to understanding of the renin-angiotensin system, the review mainly focuses on the signal transduction pathways that are induced after stimulation of the AT_1 and AT_2 receptors. The physiological properties, the localisation and the regulation of these receptors are discussed. In the second part of the review the role of the angiotensin receptors in various cardiovascular diseases, such as hypertension, atherosclerosis and chronic heart failure is presented.

To prevent the deleterious effects of the renin-angiotensin system, several drugs have been developed that can inhibit the effects of angiotensin II. These drugs can be classified in two groups: 1) ACE-inhibitors, that diminish the conversion from angiotensin I to angiotensin II; and 2) AT_1 receptor blockers (ARBs), drugs that inhibit the activation of AT_1 receptors by angiotensin II. The first ACE-inhibitor, captopril, was presented in 1977, and since that moment thousands of studies have proven the benefits of ACE-inhibitors in laboratory models as well as in several clinical settings. The first ARB, losartan, was described in 1988. Our knowledge of ARBs is much less extensive. In the clinical setting, ARBs are mainly used in hypertension and in heart failure. They are an alternative to ACE-inhibitors, when patients are intolerant to ACE-inhibitors.

Stimulation of the RAS, amongst others, leads to atherosclerosis, and subsequently to a significant obstruction, a so-called stenosis, of the coronary arteries. To prevent further problems of this stenosis (a myocardial infarction for example), a PTCA (percutaneous transluminal coronary angioplasty) can be performed. During this procedure, the (atherosclerotic) tissue is pushed aside with a balloon. Nowadays, placement of a stent (a

metal tube) at the place of the stenosis is usually included in the PTCA-procedure to prevent recoil of the tissue that is pushed aside. Unfortunately, in some patients cells start to grow in the stent, leading to so-called in-stent restenosis, a life-threatening disease.
The first aim of this thesis, as presented in *Chapter 2*, was to describe the (possible) role of the RAS, including the AT_1 receptor, in the pathophysiological process of in-stent restenosis (chapters 3 and 4). In the second part of this thesis our knowledge of AT_1 receptor blockers (ARBs) in the cardiovascular system is further expanded (chapters 5 and 6).

Chapter 3 describes the localisation of ACE and the AT_1 receptor in both *de-novo* stenotic lesions and in-stent restenotic lesions in human coronary arteries. Furthermore the cellular components involved in these two types of stenotic lesions were determined. For this purpose, an atherectomy catheter was used. This is a device with which intact specimens of stenotic lesions in human coronary arteries can be obtained. Biopsies of *de-novo* stenotic lesions contained several different tissue components, such as macrophages, vascular smooth muscle cells (VSMCs), sclerotic tissue and lipid cores, as might be expected from an atherosclerotic lesion. In-stent restenotic lesions, on the other hand, contained mostly VSMCs in abundant myxoid extracellular matrix, a tissue typical for neointima. We also found different amounts of macrophages. This was a surprising result, because in previous studies by others, no macrophages were detected at the same time point. In both kinds of lesions, the macrophages and the VSMCs carried the AT_1 receptor. ACE was only found in macrophages. Because in-stent restenotic lesions contained mainly VSMCs, the staining for the AT_1 receptor was more intense in these biopsies. As the amount of macrophages in in-stent restenotic lesions was much smaller, the amount of ACE was equally less than in *de-novo* lesions. These findings led to the conclusions that the renin-angiotensin system, and especially the AT1 receptor, might play an important role in the genesis of in-stent restenosis.

To further evaluate the influence of the renin-angiotensin system, we investigated whether ACE-levels before stent placement, can predict in-stent restenosis. The results of this study are written in *chapter 4*. Patients from chapter 3 with *de-novo* stenosis underwent a re-catheterisation after 12 months in order to determine the degree of in-stent restenosis in relation to the amount of ACE in the original plaque, removed with the atherectomy catheter 12 months before. In a second group of patients, the ACE activity in peripheral blood was measured before they underwent percutaneous coronary intervention with stent placement. After 8 months, adverse cardiovascular events were counted (as a measure of in-stent restenosis) and related to the ACE-activity.
In both substudies, no relation between the amount of ACE or ACE-activity and in-stent restenosis could be established. We concluded that pre-procedural ACE-activity, in plasma as well as in the atherosclerotic plaque, does not predict the occurrence of in-stent restenosis.

Since the first description of a selective AT_1 receptor blocker (ARB), several ARBs have been developed and tested extensively. Nevertheless, different ARBs have not been compared in clinical trials or in a human cardiovascular model. Furthermore, the type of antagonism

of the ARBs in human vessels has not been established yet. In the study presented in *chapter 5*, the functional type of antagonism of the ARBs candesartan, losartan, valsartan and EXP 3174 (an active metabolite of losartan) is described.
It appeared that losartan acts as a surmountable antagonist. This means that independent of the concentration of losartan, high concentration of the agonist, i.e. angiotensin II, can always overcome the antagonist and thereby still activate the AT_1 receptor. Valsartan and candesartan on the other hand act as insurmountable antagonists, meaning that even high concentrations of angiotensin II can never activate a receptor to which the ARB is bound. Also EXP 3174 has insurmountable properties. The degree of receptor blockade by candesartan, EXP 3174 and valsartan in this model is equal in the maximum concentration used, and more effective than by losartan.

Whereas the beneficial effects of ACE-inhibitors on endothelial function are well-known, the influence of ARBs on the endothelium is less investigated. In the study presented in *chapter 6*, the effects of the ARB candesartan on endothelial function in a rat model of chronic heart failure is tested, and compared to the ACE-inhibitor lisinopril. Chronic heart failure in the rats was induced by myocardial infarction (MI) after coronary ligation. Endothelial function was determined by measuring acetylcholine (ACh)-induced, i.e. endothelium-dependent, vasodilation in aortic rings. This vasodilation was diminished in untreated rats with chronic heart failure, compared with control rats. Both lisinopril and candesartan fully normalised ACh-induced dilation, and thus the endothelial function, in a comparable way.

GENERAL DISCUSSION

The first aim of this thesis was to elucidate the role of the renin-angiotensin system in the process of in-stent restenosis. We demonstrated that essential parts of the RAS, namely ACE and the AT_1 receptor, are present in in-stent restenosis, and that in-stent restenotic lesions mainly consist of VSMCs. Since stimulation of the AT_1 receptor by angiotensin II leads to growth and migration of VSMCs, it is likely that this process indeed is driven by the renin-angiotensin system.
Recently, a study has been published, in which the use of ACE-inhibitors after stent placement even increased the incidence of in-stent restenosis.[1] This study confirms our findings presented in chapter 4, that pre-procedural ACE-activity does not predict the occurrence of in-stent restenosis. The lack of relation between ACE-activity and in-stent restenosis, however, does not conflict with our theory about the influence of the RAS on in-stent restenosis. Via several mechanisms, angiotensin II can still be produced, for instance via chymase (another angiotensin converting enzyme), or via locally active ACE, not inhibited by ACE-inhibitors. Therefore we presume that ARBs, in contrast to ACE-inhibitors should be able to diminish the occurrence of in-stent restenosis. This concept already has been

proven in a small open-label study.[2] Future studies will have to be performed to test the hypothesis that ARBs, either applied locally via the stent or as oral drugs, are able to prevent in-stent restenosis.

At present, ARBs are used as a drug that can be prescribed as an alternative for ACE-inhibitors. Indeed we showed that ARBs are as useful as ACE-inhibitors in ameliorating endothelial dysfunction in a model of chronic heart failure. Also several clinical studies have proven that ARBs are not inferior to ACE-inhibitors. More than that, ARBs are the only drugs that have proven to be better than beta-blockers in the treatment of patients with hypertension.[3] Whether one ARB should be preferred above another, is not clear. We did not find significant differences in a pharmacological comparison between candesartan, valsartan and the active metabolite of losartan.

Another theoretical advantage of ARBs over ACE-inhibitors is that by selectively blocking the AT_1 receptor, an upregulation of angiotensin II will take place. In this situation, this will lead to selective activation of the AT_2 receptor. As can be read in chapter 1, the AT_2 receptor opposes the deleterious effects of the AT_1 receptor. This possible beneficial role of the AT_2 receptor in the human cardiovascular system is not proven yet. Especially the discussion whether the AT_2 receptor is able to induce vasodilation in human arteries (as it does in some animal models) has not been finished.

In conclusion, this thesis provides evidence that the renin-angiotensin system is involved in the genesis of in-stent restenosis. It is likely that especially the AT_1 receptor is involved in this process, and future research should aim at the possibility that angiotensin II receptor blockers are effective in preventing in-stent restenosis.

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Chapter 8

Nederlandse samenvatting

In de prehistorie, toen er nog werd gejaagd met mes en speer, hing het leven af van een snelle reactie op eventuele verwondingen. Als iemand werd verwond door een gevaarlijk roofdier, moest het lichaam adequaat kunnen reageren op het plotselinge bloedverlies. Het renine-angiotensine systeem zorgde hiervoor. Activatie van dit systeem na een verwonding veroorzaakt een snel samenknijpen van bloedvaten en activeert andere systemen om het bloedverlies te stelpen. Daarnaast wordt de hoeveelheid vocht in de bloedbaan op peil gebracht door de nieren te beïnvloeden om minder urine te produceren. Tenslotte bevordert het renine-angiotensine systeem het herstel van de verwonde bloedvaten door vorming van bindweefsel, vaatwandverdikking en activatie van ontstekingscellen.
Maar de tijden zijn veranderd. Een snelle reactie is nog steeds van belang, maar in onze huidige maatschappij wordt het renine-angiotensine systeem continu geactiveerd als reactie op de bekende risicofactoren voor hart- en vaatziekten, zoals roken, hoge bloeddruk, te hoog cholesterol en suikerziekte. Het geactiveerde renine-angiotensine systeem speelt een doorslaggevende rol in het ontstaan van vaatvernauwing, doordat het beschadiging van bloedvaten en weefsels, hoge bloeddruk en andere hart- en vaatziekten in de hand werkt. Als gevolg hiervan is het renine-angiotensine systeem tegenwoordig niet allen een vriend, maar ook een vijand geworden.

Het renine-angiotensine systeem bestaat uit een cascade van eiwitten. De belangrijkste hiervan staan in figuur 1 van hoofdstuk 1. De stappen van de cascade die in dit proefschrift een rol spelen, zijn in de eerste plaats de productie van angiotensine II uit angiotensine I, en in de tweede plaats de activatie van de angiotensine II receptoren.

De omzetting van angiotensine I naar angiotensine II kan door verschillende bioactieve stoffen veroorzaakt worden. De belangrijkste hiervan is ACE (Angiotensin Converting Enzyme). Deze omzetting gebeurt niet alleen in het bloed, maar ook in andere soorten weefsels, waaronder de vaatwand en het hart.
Angiotensine II is een zeer actieve stof, die effecten op de vaatwand, het hart en vele andere organen uitoefent via binding aan de zogenaamde angiotensine II receptoren, waarvan bij mensen met name de type 1 en 2 (AT_1 en AT_2) receptoren een rol spelen. Hierbij veroorzaakt de AT_1 receptor de ongewenste effecten van het renine-angiotensine systeem, en gaat de AT_2 receptor deze effecten weer tegen. Blokkade van de AT_1 receptor, met zogenaamde AT_1 receptor blokkers, is dan ook een effectieve methode om ziektes waarbij het renine-angiotensine systeem een rol speelt af te remmen.

In *Hoofdstuk 1* wordt de rol van de angiotensine receptoren in het (menselijke) cardiovasculaire systeem uiteen gezet. Hierbij wordt de nadruk gelegd op de systemen die in de cel geactiveerd worden na stimulatie van de angiotensine receptoren. Ook worden de lokalisatie en regulatie van deze receptoren besproken. In het tweede deel van dit hoofdstuk wordt de rol van de angiotensine receptoren in verschillende hart- en vaatziekten, zoals hoge bloeddruk en chronisch hartfalen, belicht.

Eén van de belangrijkste bloedvaten binnen de cardiologie is de kransslagader, die de hartspier van bloed voorziet. Vernauwingen ('stenoses') in deze bloedvaten kunnen dan ook leiden tot allerlei hartziekten, waaronder een hartinfarct. Een veelgebruikte therapie voor een stenose van de kransslagaderen is een Dotter-procedure ('PTCA'), waarbij de vernauwing met behulp van een ballon wordt weggedrukt. Dit is een effectieve behandeling gebleken, maar in sommige gevallen (tot 30%) treedt er toch weer opnieuw een vernauwing ('restenose') op dezelfde plaats op. Een van de oorzaken hiervoor is dat het weggedrukte materiaal weer terugveert. Om die reden wordt tijdens de Dotter-procedure steeds vaker een stent geplaatst. Dit is een hol buisje, dat ter plaatse van de stenose wordt gezet, zodat het terugveren van de verdikte vaatwand niet meer mogelijk is. Dit heeft tot een sterke verbetering van het lange-termijnsresultaat van dotteren geleid. Helaas groeien deze stents toch weer dicht bij sommige patiënten (10-20%). Dit wordt 'in-stent restenose' genoemd. De hoofdstukken 3 en 4 gaan over de relatie tussen het renine-angiotensine systeem en in-stent restenose.

In *Hoofdstuk 3* worden de lokalisaties in menselijke kransslagaderen van ACE en de AT_1 receptor beschreven in zowel in-stent restenoses als in nieuwe stenoses (d.w.z. vernauwingen, die nooit eerder zijn behandeld). Daarnaast worden ook de verschillende soorten cellen die de (re-)stenoses vormen benoemd.
Voor dit onderzoek is materiaal uit stenoses en in-stent restenoses uit menselijke kransslagaders verkregen met behulp van een 'atherectomie-catheter'. Met dit apparaat, dat op een appelboor lijkt, is het mogelijk om stenoses uit kransslagaderen te boren op een zodanige manier dat het verkregen materiaal mooi intact blijft voor verder onderzoek.
Zoals bekend uit vroegere onderzoeken, bevatten de weefselstukjes uit nieuwe stenoses veel verschillende celtypes, zoals gladde spiercellen, macrofagen (een type ontstekingscellen), bindweefsel en vetophopingen. In-stent restenoses blijken daarentegen eigenlijk bijna alleen maar te bestaan uit gladde spiercellen temidden van overvloedig aanwezig extracellulaire slijmerige materie. In deze materie vonden wij ook wisselende hoeveelheden macrofagen. Dit is verrassend, omdat voorheen werd beschreven dat ontstekingsprocessen na ongeveer een maand niet meer aanwezig zijn en onze weefselmonsters uit in-stent restenoses zijn meer dan een maand na plaatsing van de stent verzameld.
In beide typen stenoses vonden we de AT_1 receptor in macrofagen en gladde spiercellen. ACE vonden wij alleen in macrofagen. Aangezien de in-stent restenoses bijna alleen maar bestonden uit gladde spiercellen, werd de AT_1 receptor in deze weefsels overal gevonden, in tegenstelling tot ACE, dat alleen in de spaarzaam aanwezige macrofagen werd gevonden. Bovenstaande bevindingen doen vermoeden dat het renine-angiotensine systeem en met name de AT_1 receptor, een belangrijke rol spelen bij het ontstaan van in-stent restenose.

Om de invloed van het renine-angiotensine systeem op het ontwikkelen van in-stent restenose verder te bestuderen, onderzochten we ook of de hoeveelheid ACE op het moment van de plaatsing van de stent het ontstaan van in-stent restenose zou kunnen voorspellen. De resultaten van dit onderzoek zijn in *Hoofdstuk 4* beschreven.

Een aantal van de patiënten beschreven in hoofdstuk 3 met een nieuwe stenose werden na een jaar opnieuw onderzocht d.m.v. een hartcatheterisatie. Op deze manier kon bekeken worden of er in de stent opnieuw weefsel was gegroeid, en zo ja, hoeveel. Dit is dan vergeleken met de hoeveelheid ACE die bij het eerste onderzoek in de stenose werd gevonden. In een tweede groep patiënten werd de ACE-activiteit in het bloed bepaald voor het plaatsen van de stent. Acht maanden later werd gekeken hoeveel van deze patiënten opnieuw klachten kregen, die kunnen wijzen op in-stent restenose. Het optreden van klachten werd weer gerelateerd aan de hoeveelheid ACE in het bloed.
In beide onderzoeken kon geen relatie worden aangetoond tussen de hoeveelheid ACE en het optreden van in-stent restenose. Daarom concludeerden wij dat de hoeveelheid ACE, zowel in het bloed als in het weefsel, niet voorspellend is voor het optreden van in-stent restenose. Dit is in overeenstemming met het feit dat in in-stent restenose-weefsel nauwelijks ACE werd gevonden.

Om de ongewenste effecten van het renine-angiotensine systeem te voorkomen, zijn er verschillende soorten geneesmiddelen ontwikkeld. Deze kunnen in twee groepen worden ingedeeld: ACE-remmers en AT_1 receptor blokkers (ARBs). ACE-remmers verminderen de productie van angiotensine II, door het enzym ACE te remmen. ARBs blokkeren de activatie van de AT_1 receptoren door angiotensine II.
ACE-remmers bestaan al meer dan 25 jaar, en zijn dan ook uitgebreid onderzocht. Hun effectiviteit wat betreft het voorkomen en behandelen van hart- en vaatziekten is in zowel in het laboratorium als in klinisch onderzoek uitgebreid bewezen. De eerste ARB, losartan, is voor het eerst in 1988 beschreven. Onze kennis over de ARBs is dan ook veel minder uitgebreid. ARBs worden in de dagelijkse praktijk al gebruikt bij patiënten met hoge bloeddruk en met hartfalen, maar alleen als alternatief bij die patiënten, die overgevoelig zijn voor ACE-remmers.

Sinds de eerste beschrijving van een ARB, zijn er steeds meer ontwikkeld. Desondanks is er nooit vergelijkend onderzoek gedaan naar de effectiviteit van de verschillende ARBs, noch in een klinisch onderzoek, noch in een humaan model van hart- en vaatziektes. Daarnaast is ook de manier waarop de ARBs de AT_1 receptor blokkeren in menselijke bloedvaten nooit onderzocht.
In theorie kunnen geneesmiddelen receptoren op twee manieren blokkeren. De eerste heet 'overkomelijk'. Dit betekent dat de blokkering van de receptor (in dit geval de AT_1 receptor) door een geneesmiddel altijd kan worden overtroffen door de normale activator van de receptor (in dit geval angiotensine II) als de concentratie maar hoog genoeg is. Bij de aanwezigheid van een 'onoverkomelijk' geneesmiddel zal de normale activator, door wat voor mechanisme dan ook, nooit aan de receptor binden.

Hoofdstuk 5 beschrijft het mechanisme waarmee de ARBs candesartan, losartan, valsartan en EXP3176 (een metaboliet van losartan, die ook als ARB werkt) de angiotensine receptoren blokkeren in menselijke vaten. Het blijkt dat losartan een overkomelijke blokkeerder

is, en dat de andere blokkeerders van het onoverkomelijke type zijn. Het lijkt tenslotte dat de mate waarin EXP3176, candesartan en valsartan de AT_1 receptor blokkeren even groot is, terwijl losartan zelf minder blokkerend vermogen heeft.

In *Hoofdstuk 6* is de invloed van ARBs op het endotheel, de binnenste laag van de bloedvaten onderzocht. Dit endotheel speelt een belangrijke rol in het in balans houden van allerlei invloeden op de bloedvaten. Over de gunstige effecten van ACE-remmers op het endotheel is veel bekend, in tegenstelling tot de invloed van ARBs op het endotheel. In dit onderzoek is het effect van de ARB candesartan op de endotheelfunctie in een rattenmodel van hartfalen vergeleken met het effect van de ACE-remmer lisinopril. De endotheelfunctie werd gemeten in de aorta van deze ratten, nadat ze hartfalen hadden gekregen. Zoals verwacht was de endotheelfunctie sterk verslechterd in ratten met hartfalen in vergelijking met controle ratten. Zowel candesartan als lisinopril verbeterde de endotheelfunctie weer tot het niveau van de controle ratten.

Een van de doelen van dit proefschrift was om de rol van het renine-angiotensine systeem bij het ontstaan van in-stent restenose te verduidelijken. Wij toonden aan dat de AT_1 receptor aanwezig is in in-stent restenose. Aangezien stimulatie van de AT_1 receptor leidt tot proliferatie van gladde spiercellen, die het voornaamste bestandsdeel vormen van in-stent restenose, is het waarschijnlijk dat het renine-angiotensine systeem belangrijk is in de ontwikkeling van in-stent restenose.
Het feit dat de hoeveelheid ACE niet voorspellend is voor in-stent restenose is hier niet mee in tegenspraak. Zoals te zien is in figuur 1 van hoofdstuk 1, zijn er meer mechanismen die de productie van angiotensine II kunnen veroorzaken. Daarom is het aannemelijk dat ARBs, die activatie van de AT_1 receptoren voorkomen, het ontstaan van in-stent restenose kunnen afremmen, in tegenstelling tot ACE-remmers. Deze theorie is al bevestigd in één kleine klinische studie, maar nog zal moeten worden bewezen in grotere klinische studies.

Tegenwoordig worden ARBs gezien als een alternatief voor ACE-remmers voor de behandeling van diverse hart- en vaatziektes. Wij toonden aan dat ARBs even effectief zijn als ACE-remmers wat betreft het verbeteren van de endotheelfunctie bij ratten met hartfalen. Verschillende onderzoeken hebben bewezen dat ARBs niet onderdoen voor ACE-remmers bij verschillende ziektebeelden. Of de ene ARB moet worden verkozen boven een andere is nog niet duidelijk. Wij vonden in ieder geval geen duidelijke farmacologische verschillen tussen de ARBs candesartan, valsartan en EXP3174.

Concluderend verschaft dit proefschrift bewijzen dat het renine-angiotensine systeem betrokken is bij het ontstaan van in-stent restenose. Het is waarschijnlijk dat de AT_1 receptor betrokken is bij dit proces. Toekomstig onderzoek zal moeten aantonen of AT_1 receptor blokkers effectief zijn in het voorkomen van in-stent restenose.

Chapter 9

Dankwoord

Zoals bekend is het voltooien van een proefschrift niet alleen het werk van degene wiens naam op de kaft staat. Sterker nog, voor mijn gevoel was ik slechts een radertje in een goed lopend systeem, waarbij de input van zeer vele kanten kwam. Er zijn dan ook vele personen die ik wil bedanken, maar er zijn drie mensen die ik als eerste wil noemen, omdat ze zeer belangrijk zijn geweest in mijn "Groningse periode". Dit zijn Adriaan Voors, Azuwerus van Buiten en Greetje de Jong.

Adriaan Voors heeft mij geholpen aan mijn onderzoeksplek in Groningen en was ook nog een steun in de rug bij mijn sollicitatie in Nieuwegein. Hij heeft dus een belangrijke rol in mijn carrièreplanning gespeeld. Maar ook bij mijn onderzoek heeft hij een zeer actieve rol gespeeld. Zowel als klankbord voor mijn ideeën, als als verzinner van nieuwe plannen. Het is niet voor niets dat hij mede-auteur is bij bijna alle artikelen in dit proefschrift. Ik hoop dan ook dat we in de toekomst nog veel kunnen blijven samenwerken, en ik bel je bij toekomstige carrière-moves.

Azuwerus van Buiten was (en is) op het laboratorium mijn steun en toeverlaat. De hoeveelheid experimenten die hij voor mij heeft uitgevoerd grenst aan het onwaarschijnlijke. Dat hij hier altijd vrolijk bij bleef, en zelfs mijn experimenteer-vaardigheden ontspannen wist te begeleiden is welhaast nog onwaarschijnlijker. Veel dank dan ook hier voor. Nog een koffie?

Greetje de Jong heeft weliswaar niet veel te maken gehad met het onderzoek dat in dit proefschrift is beschreven, maar des te meer met het dagelijkse leven in de Greenhouse. Niet alleen was zij zeer betrokken bij de verschillende klinische studies die ik deed, maar ook was zij de dagelijkse steun en toeverlaat. Volgens Greenhouse-collega's kon ik de dag niet beginnen, zonder even met Greetje te hebben gebeld, en dat was denk ik ook zo. Wanneer verhuis je nou naar het Westen?

Met Wiek van Gilst heb ik een ideale promotor getroffen. Steeds weer wist hij ervoor te zorgen dat ik er vertrouwen in hield dat het wel goed zal komen met mijn proefschrift. Wiek is op wetenschappelijk gebied een zeer goede stimulator en criticus. Voor verschillende onoplosbare problemen blijkt hij binnen de kortste keren een oplossing te hebben. Niet alleen wetenschappelijk, maar ook sociaal is Wiek een geweldige promotor. Ik koester vele goede herinneringen aan onze uitstapjes in binnen- en buitenland. Wanneer is de volgende pasta-party?

Ook mijn tweede promotor, Dirk-Jan van Veldhuisen, wil ik hartelijk bedanken voor het werk dat hij heeft verricht om mijn promotie tot een goed einde te brengen. En nog een goede kijk op voetbal ook!

Ad van Boven begeleidde mij op de dagelijkse werkvloer. Hij was de grote stimulator van het 'atherectomie-onderzoek' dat heeft geleid tot twee hoofdstukken in dit proefschrift. Daarnaast is ook Ad iemand, die de gezelligheid van en na het werk hoog in het vaandel heeft staan. Kortom een zeer gewaardeerde co-promotor!

Hendrik Buikema heeft mij ingewijd in en enthousiast gemaakt voor de biologie van de vaatwand. Zijn kritische blik op mijn experimenten en plannen waren zeer leerzaam. Net als onze zomeravond-discussies over sport en andere belangrijke zaken overigens.

De leden van de Leescommissie, Piet Boonstra, Pieter de Graeff en Thijs Plokker, wil ik bedanken voor hun kritische blik op mijn werk. Piet Boonstra bedank ik verder voor de prettige samenwerking met de afdeling thoraxchirurgie, waardoor wij honderden LIMA's hebben kunnen onderzoeken. Thijs Plokker wil ik ook bedanken voor zijn hulp bij het onderzoek dat heeft geleid tot hoofdstuk 4 en voor de beschouwende supervisies op de eerste hart hulp.

De collegae in de Greenhouse (en enkelen in de buitengewesten) bepalen voor het grootste deel het dagelijks leven van de promovendus. Het was erg leuk om in zo'n gezellige en collegiale groep te mogen meedraaien. Met name binnen de flippergroep (Joost van Melle en Richard de Jong) was de collegialiteit zo intens, dat dit de voortvarendheid van het onderzoek niet altijd ten goede kwam. Ook Folkert Asselbergs wil ik erg bedanken. Niet alleen omdat je mij als paranimf wilt ondersteunen, maar ook je vrolijke, relativerende kijk op alles wat het leven belangrijk maakt doet mij vaak zeer goed. Anton Roks, Gilles Diercks en ook Richard wil ik bedanken dat ik als paranimf mocht oefenen voor mijn eigen promotie! Tot slot bedankt Paul van Haelst, Rudolf de Boer, Hans-Marc Siebelink, Geert Tjeerdsma, Stephan Monnink, Anton Tuinenburg, Bas Schoonderwoerd, Femke Niewold-Verdoes, Trudeke van Noord, Vincent Hagens, Titia Spijkerman, Peter-Paul van Geel en Bas Langeveld.

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Lieve pappa en mamma. Jullie onvoorwaardelijke steun vormt de basis van dit proefschrift. Het blijft bijzonder hoe jullie drie zulke verschillende kinderen hebben kunnen opvoeden, die het ook nog zo goed met elkaar kunnen vinden! Het vervult me telkens weer met trots wanneer ik te horen krijg dat ik zo op een van jullie lijk. Dit proefschrift is niet voor niets aan jullie opgedragen.

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